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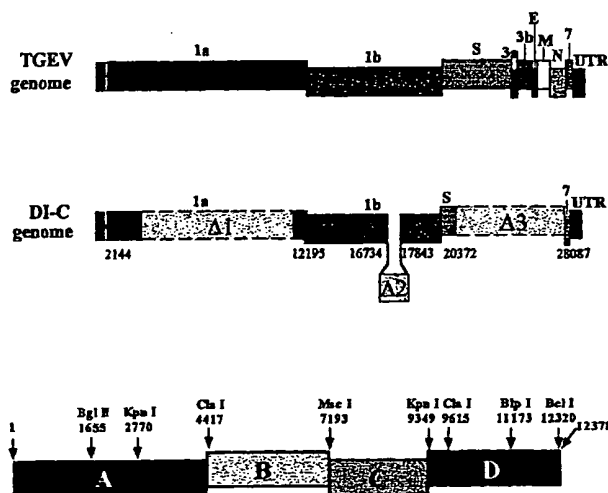
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(54) Title: INFECTIOUS CLONES



(57) Abstract: The present invention relates to methods of preparing a DNA comprising steps, wherein (a) a DNA comprising a full length copy of the genomic RNA (gRNA) or an RNA virus; or (b) a DNA comprising one or several fragments of a gRNA of an RNA virus, which fragments code for an RNA dependent RNA polymerase and at least one structural or non-structural protein; or (c) a DNA having a homology of at least 60% to the sequences of (a) or (b); is cloned into a bacterial artificial chromosome (BAC). Additionally, DNAs are provided, which comprise sequences derived from the genomic RNA (gRNA) of a coronavirus which sequences have a homology of at least 60% to the natural sequence of the virus and code for an RNA dependent RNA polymerase and at least one structural or non-structural protein, wherein a fragment of said DNA is capable of being transcribed into RNA which RNA can be assembled to a virion. Further, the use of these nucleic acids for preparation of viral RNA or virions as well as pharmaceutical preparations comprising these DNAs, viral RNAs or virions is disclosed.

## Infectious Clones

### FIELD OF THE INVENTION

This invention relates to methods of preparing a DNA or an RNA, nucleic acids obtainable by this method and their use as vaccines and for gene therapy.

### BACKGROUND OF THE INVENTION

Advances in recombinant DNA technology have led to progress in the development of gene transfer between organisms. At this time, numerous efforts are being made to produce chemical, pharmaceutical, and biological products of economic and commercial interest through the use of gene transfer techniques.

One of the key elements in genetic manipulation of both prokaryotic and eukaryotic cells is the development of vectors and vector-host systems. In general, a vector is a nucleic acid molecule capable of replicating or expressing in a host cell. A vector-host system can be defined as a host cell that bears a vector and allows the genetic information it contains to be replicated and expressed.

Vectors have been developed from viruses with both DNA and RNA genomes. Viral vectors derived from DNA viruses that replicate in the nucleus of the host cell have the drawback of being able to integrate into the genome of said cell, so they are generally not very safe. In contrast, viral vectors derived from RNA viruses, which replicate in the cytoplasm of the host cell, are safer than those based on DNA viruses, since the replication occurs through RNA outside the nucleus. These vectors are thus very unlikely to integrate into the host cell's genome.

cDNA clones have been obtained from single-chain RNA viruses with positive polarity [ssRNA(+)], for example, picornavirus (Racaniello & Baltimore, 1981); bromovirus (Ahlquist et al., 1984); alphavirus, a genus that includes the Sindbis virus; Semliki Forest virus (SFV) and the Venezuelan equine encephali-

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tis virus (VEE) (Rice et al., 1987; Liljeström and Garoff, 1991; Frolov et al., 1996; Smerdou and Liljeström, 1999); flavivirus and pestivirus (Rice and Strauss, 1981; Lai et al., 1991; Rice et al., 1989); and viruses of the *Astroviridae* family (Geigenmüller et al., 1997). Likewise, vectors for the expression of heterologous genes have been developed from clones of DNA complementary to the genome of ssRNA(+) virus, for example alphavirus, including the Sindbis virus, Semliki Forest virus (SFV), and the Venezuelan equine encephalitis (VEE) virus (Frolov et al., 1996; Liljeström, 1994; Pushko et al., 1997). However, all methods of preparing recombinant viruses starting from RNA viruses are still complicated by the fact that most of the viruses comprise sequences which are toxic for bacteria. Preparing a cDNA of the viral RNA and subcloning of the cDNA in bacteria therefore often leads to deletion or rearrangement of the DNA sequences in the bacterial host. For this purpose most of the commonly used subcloning and expression vectors cannot be used for preparation of large DNA sections derived from recombinant RNA viruses. However, obtaining vectors, which can carry long foreign DNA sequences is required for a number of aspects in the development of pharmaceuticals, specifically vaccines.

The coronaviruses are ssRNA(+) viruses that present the largest known genome for an RNA virus, with a length comprised between about 25 and 31 kilobases (kb) (Siddell, 1995; Lai & Cavanagh, 1997; Enjuanes et al., 1998). During infection by coronavirus, the genomic RNA (gRNA) replicates and a set of subgenomic RNAs (sgRNA) of positive and negative polarity is synthesized (Sethna et al., 1989; Sawicki and Sawicki, 1990; van der Most & Spaan, 1995). The synthesis of the sgRNAs is an RNA-dependent process that occurs in the cytoplasm of the infected cell, although its precise mechanism is still not exactly known.

The construction of cDNAs that code defective interfering (DI) genomes (deletion mutants that require the presence of a complementing virus for their replication and transcription) of some coronaviruses, such as the murine hepatitis virus (MHV),

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infectious bronchitis virus (IBV), bovine coronavirus (BCV) (Chang et al., 1994), and porcine gastroenteritis virus (TGEV) (Spanish Patent Application P9600620; Méndez et al., 1996; Izeta et al., 1999; Sánchez et al., 1999) has been described. However, the construction of a cDNA clone that codes a complete genome of a coronavirus has not been possible due to the large size of and the toxic sequences within the coronavirus genome.

In summary, although a large number of viral vectors have been developed to replicate and express heterologous nucleic acids in host cells, the majority of the known vectors for expression of heterologous genes are not well suited for subcloning of RNA viruses. Further, the viral vectors so obtained present drawbacks due to lack of species specificity and target organ or tissue limitation and to their limited capacity for cloning, which restricts the possibilities of use in both basic and applied research.

Hence there is a need for methods to develop new vectors for expression of heterologous genes that can overcome the aforesaid problems. In particular, it would be advantageous to have large vectors for expression of heterologous genes with a high level of safety and cloning capacity, which can be designed so that their species specificity and tropism can be controlled.

#### SUMMARY OF THE INVENTION

According to the present invention the above problems are solved by a method of preparing a DNA comprising steps, wherein

- (a) a DNA comprising a full length copy of the genomic RNA (gRNA) of an RNA virus; or
- (b) a DNA comprising one or several fragments of a gRNA of an RNA virus, which fragments code for an RNA dependent RNA polymerase and at least one structural or non-structural protein; or
- (c) a DNA having a homology of at least 60% to the sequences of (a) or (b);

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is cloned into a bacterial artificial chromosome (BAC).

Surprisingly, the present inventors found that the problems encountered by the prior art methods to subclone and express large DNA sequences derived from viral gRNA can be overcome by using BACs as a cloning vector. The use of BACs has the particular advantage that these vectors are present in bacteria in a number of one or two copies per cell, which considerably limits the toxicity and reduces the possibilities of interplasmid recombination.

The invention further provides methods of preparing a viral RNA or a virion comprising steps, wherein a DNA is prepared according to one of the above methods, the DNA is expressed and the viral RNA or the virion is isolated. Further, methods of preparing pharmaceuticals, specifically vaccines comprising the steps of the above methods to prepare a DNA are disclosed.

According to another aspect of the present invention provides a DNA comprising sequences derived from the genomic RNA (gRNA) of a coronavirus which sequences have a homology of at least 60% to the natural sequence of the coronavirus and code for an RNA dependent RNA polymerase and at least one structural or non-structural protein, wherein a fragment of said DNA is capable of being transcribed into RNA and which RNA can be assembled to a virion. The present invention also encompasses methods of preparing respective DNAs.

The present invention further provides vectors, more specifically bacterial artificial chromosomes (BACs) comprising respective nucleic acids. According to a further embodiment the present invention is directed to host cells and infectious, attenuated or inactivated viruses comprising the DNAs or RNAs of the present invention.

The invention also provides pharmaceutical preparations, such as mono- or multivalent vaccines comprising nucleic acids, vectors,

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host cells or virions of the present invention.

Finally, the present invention provides methods for producing a virion or a viral RNA comprising steps, wherein a DNA according to the present invention is transcribed and the virions or viral RNAs are recovered, as well as viral RNAs obtainable by this method.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the construction of a cDNA clone that codes an infective RNA of TGEV. Figure 1A shows the genetic structure of the TGEV, with the names of the genes indicated by letters and numbers (1a, 1b, S, 3a, 3b, E, M, N, and 7). Figure 1B shows the cDNA-cloning strategy, which consisted in completing the DI-C genome. Deletions  $\Delta 1$ ,  $\Delta 2$ , and  $\Delta 3$  that have been completed to reestablish the full length of the cDNA are indicated. The numbers located beneath the structure of the DI-C genome indicate the nucleotides that flank each deletion in said DI-C genome. Figure 1C shows the four cDNA fragments constructed to complete deletion  $\Delta 1$  and the position of the principal restriction sites used during joining. The insertion of fragment  $\Delta 1$  produced an increase in the toxicity of the cDNA.

Figure 2 shows the structure of the pBeloBAC plasmid (Wang et al., 1997) used in cloning the infective cDNA of TGEV. The pBeloBAC plasmid was provided by H. Shizuya and M. Simon (California Institute of Technology) and includes 7,507 base pairs (bp) that contain the replication origin of the F factor of *E. coli* (oriS), the genes necessary to keep one single copy of the plasmid per cell (parA, parB, parC, and repE), and the chloramphenicol-resistance gene ( $CM^r$ ). The positions of the T7 and SP6 promoters and of the unique restriction sites are indicated. CosN: site cosN of lambda to facilitate the construction of the pBAC plasmid; lac Z:  $\beta$ -galactosidase gene. Sequence loxP used during the generation of the plasmid is also indicated.

Figure 3 shows the structure of the basic plasmids used in the

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construction of TGEV cDNA. The pBAC-TcDNA<sup>ΔClaI</sup> plasmid contains all the information of the TGEV RNA except for one ClaI-ClaI fragment of 5,198 bp. The cDNA was cloned under the immediately early (IE) promoter of expression of cytomegalovirus (CMV) and is flanked at the 3'-end by a poly(A) tail with 24 residues of A, the ribozyme of the hepatitis delta virus (HDV), and the termination and polyadenylation sequences of bovine growth hormone (BGH). The pBAC-B+C+D5' plasmid contains the ClaI-ClaI fragment required to complete the pBAC-TcDNA<sup>ΔClaI</sup> until the cDNA is full length. The pBAC-TcDNA<sup>FL</sup> plasmid contains the full-length cDNA of TGEV. SAP: shrimp alkaline phosphatase.

Figure 4 shows the differences in the nucleotide sequence of the S gene of the clones of TGEV PUR46-MAD (MAD) and C11. The numbers indicate the positions of the substituted nucleotides, considering as nucleotide one of each gene the A of the initiating codon. The letters within the bars indicate the corresponding nucleotide in the position indicated. The letters located beneath the bars indicate the amino acid (aa) substitutions coded by the nucleotides that are around the indicated position. Δ6 nt indicates a 6-nucleotide deletion. The arrow indicates the position of the termination codon of the S gene.

Figure 5 shows the strategy followed to rescue the infective TGEV from the full-length TGEV cDNA. The pBAC-TcDNA<sup>FL</sup> plasmid was transfected to ST cells (pig testicle cells), and 48 h after transfection, the supernatant was used to infect new ST cells. The virus was passed at the times indicated. At each passage, aliquots of supernatant and of cellular monolayer were collected for virus titration and isolation of RNA for RT-PCR analysis, respectively. vgRNA: full-length viral RNA.

Figure 6 shows the cytopathic effect (CPE) produced by the TGEV cDNA in the transfected ST cells. The absence of CPE in non-transfected (control) ST cells (Figure 6A) and the CPE observed 14 and 20 h after transfection with pBAC-TcDNA<sup>FL</sup> in ST cells are shown (Figures 6B and 6C, respectively).

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Figure 7 shows the evolution of the viral titer with the passage. A graph representing the viral titer in the supernatant of two series of cellular monolayers (1 and 2) at different passages after transfection with pBAC-TcDNA<sup>FL</sup> is shown. Mock 1 and 2 refer to nontransfected ST cells. TcDNA 1 and 2 refer to ST cells transfected with pBAC-TcDNA<sup>FL</sup>.

Figure 8 shows the results of the analysis of the sequence of the virus recovered after transfecting ST cells with pBAC--TcDNA<sup>FL</sup>. The structure of the TGEV genome is indicated at the top of the figure. Likewise, the differences in the sequence of nucleotides (genetic markers) between the virus recovered from the pBAC-TcDNA<sup>FL</sup> (TcDNA) plasmid, and TGEV clones C8 and C11 are indicated. The positions of the differences between the nucleotides are indicated by the numbers located over the bar. The cDNA sequences of the TcDNA virus and of clone C11 were determined by sequencing the fragments obtained by RT-PCR (reverse-transcription and polymerase chain reaction). The sequence of clone C8 is being sent for publication (Penzes et al., 1999) and is included at the end of this patent. The restriction patterns are shown with ClaI and DraIII of the fragments obtained by RT-PCR that include nucleotides 18,997 and 20,990 of the TcDNA and C8 viruses. The restriction patterns show the presence or absence of ClaI and DraIII sites in the cDNA of these viruses. The result of this analysis indicated that the TcDNA virus recovered had the S-gene sequence expected for isolate C11. MWM: molecular weight markers.

Figure 9 shows the results of the RT-PCR analysis of the virus recovered. The viral RNA was expressed under the control of the CMV promoter recognized by the cellular polymerase pol II. In principle, this RNA could undergo splicing during its transport to the cytoplasm. To study whether this was the case, the sites of the RNA with a high probability of splicing were determined using a program for predicting splicing sites in sequences of human DNA (Version 2.1.5.94, Department of Cell Biology, Baylor College of Medicine) (Solovyev et al., 1994). The potential



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splicing site with maximum probability of cut had the donor site at nt 7,243 and the receiver at nt 7,570 (Figure 9A). To study whether this domain had undergone splicing, a RT-PCR fragment flanked by nt 7,078 and nt 7,802 (Figure 9B) was prepared from RNA of passages 0 and 2 of nontransfected cultures (control), or from ST cells transfected with TcDNA with the ClaI fragment in reverse orientation (TcDNA<sup>FL(-ΔClaI)RS</sup>), or in the correct orientation (TcDNA<sup>FL</sup>), and the products resulting from the RT-PCR were analyzed in agarose gels. The results obtained are shown in Figures 9C (passage 0) and 9D (passage 2).

Figure 10 shows the results of the immunofluorescence analysis of the virus produced in cultures of ST cells transfected with TcDNA. Staining for immunofluorescence was done with antibodies specific for the TGEV PUR46-MAD isolate, and for the virus recovered after transfection with the pBAC-TcDNA<sup>FL</sup> plasmid. For this, TGEV-specific monoclonal antibodies were used which bind to both isolates or only to PUR46-MAD (Sánchez et al., 1990). The result confirmed that the TcDNA virus had the expected antigenicity. The specific polyclonal antiserum for TGEV bound to both viruses, but not to the uninfected cultures, and only the expected monoclonal antibodies specific for the S (ID.B12 and 6A.C3), M (3B.B3), and N (3B.D8) proteins bound to the TcDNA virus (Sánchez et al., 1999).

Figure 11 shows the expression of GUS under different transcription-regulatory sequences (TRSs) that vary flanking region 5' of the intergenic (IG) sequence. Minigenome M39 was cloned under the control of the CMV promoter. Inserted into this minigenome was a multiple cloning sequence (PL1, 5'-CCTAGGATTTAA-ATCCTAAGG-3'; SEQ ID NO:2) and the transcription unit formed by the selected transcription-regulating sequences (TRS), another multiple cloning sequence (PL2, 5'-GCGGCCGCGCCGCGAGGCTGTCGAC-3'; SEQ ID NO:3; or PL3, 5'-GTCGAC-3'; SEQ ID NO:4), sequences with the structure of a Kozak (Kz) domain, the β-glucuronidase (GUS) gene, and another multiple cloning site (PL4, 5'-GCTAGCCCAGGCGCGGTACC-3'; SEQ ID

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NO:5). These sequences<sup>1</sup> were flanked at the 3'-end by the 3'-sequence of minigenome M39, the HDV ribozyme, and the termination and polyadenylation sequences of BGH. The TRSs had a different number (0, -3, -8, and -88) of nucleotides at the 5'-end of the IG sequence (CUAAAC)<sup>1</sup>, and came from the N, S, or M genes, as indicated. ST cells were transfected with the different plasmids, were infected with the complementing virus (PUR46-MAD), and the supernatants were passed 6 times. The GUS activity in the infected cells was determined by means of the protocol described by Izeta (Izeta et al., 1999). The results obtained by relating the GUS activity to the passage number are collected in Figure 11B.

Figure 12 shows the expression of GUS under different TRSs that vary in the 3'-flanking region of the IG sequence (see Figure 11A). Using this transcription unit with the 5'-flanking region corresponding to the -88 nt of the N gene of TGEV plus the IG sequence (CUAAAC), the 3'-flanking sequences were modified. These sequences corresponded to those of the different TGEV genes (S, 3a, 3b, E, M, N, and 7), as is indicated in Figure 12A. In two cases, 3'-sequences were replaced by others that contained a restriction site (SalI) and an optimized Kozak sequence (Kz), or by a sequence identical to the one that follows the first IG sequence located following the leader of the viral genome. The activity of GUS in the infected cells was determined by means of the protocol described above (Izeta et al., 1999). cL12 indicates a sequence of 12 nucleotides identical to that of 3'-end of the "leader" sequence of the TGEV genome (see the virus sequence indicated at the end). The results obtained by relating the expression of GUS to the passage number are collected in Figure 12B.

Figure 13 shows the effect of the site of insertion of the modu-

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<sup>1</sup> It should be noted that CTAAAC and CUAAC have the same meaning for the purpose of this patent. The first represents the sequence of the DNA and the second that of the corresponding RNA.

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le of expression in the minigenome over the levels of GUS expression. The GUS transcription unit, containing -88 nt of the N gene flanking the 5'-end of the IG sequence (CUAAAC), and the Kz sequences flanking the 3'-end (see Figure 12A), was inserted into four single restriction sites in minigenome M39 (Figure 13A) to determine if all these sites were equally permissive for the expression of the heterologous gene. ST cells were transfected with these plasmids and infected with the complementing virus (PUR46-MAD). The GUS activity in the infected cells was determined at passage 0 (P0) following the protocol described above (Izeta et al., 1999). The results obtained are collected in Figure 13B.

#### DETAILED DESCRIPTION OF THE INVENTION

According to the present invention methods of preparing a DNA are provided, which comprise steps, wherein

- (a) a DNA comprising a full length copy of the genomic RNA (gRNA) of an RNA virus; or
- (b) a DNA comprising one or several fragments of a gRNA of an RNA virus, which fragments code for an RNA dependent RNA polymerase and at least one structural or non-structural protein; or
- (c) a DNA having a homology of at least 60% to the sequences of (a) or (b);

is cloned into a bacterial artificial chromosome (BAC).

According to the present application a "bacterial artificial chromosome" is a DNA sequence which comprises the sequence of the F factor. Plasmids containing this sequences, so-called F plasmids, are capable of stably maintaining heterologous sequences longer than 300 Kb in low copy number (one or two copies per cell). Respective BACs are known in the art (Shizuya et al., 1992).

According to the present invention the DNA cloned into the BAC

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has a homology of at least 60%, preferably 75% and more preferably 85 or 95%, to a natural sequence of an RNA virus. Sequence homology is preferably determined using the Clustal computer program available from the European Bioinformatics Institute (EBI).

According to the methods of the present invention the DNA cloned into the BAC may further comprise sequences coding for several or all except one of the structural or non-structural proteins of the virus.

In a preferred embodiment of the present invention the DNA cloned into the BAC further comprises sequences encoding one or several heterologous gene. According to the present application a gene is characterized as a "heterologous gene" if it is not derived from the virus which was used as a source for the genes encoding the RNA dependent RNA polymerase and the structural or non-structural protein. A "heterologous gene" thus also refers to genes derived from one type of virus and expressed in a vector comprising sequences derived from another type of virus. Any heterologous gene of interest can be inserted into the nucleic acids of the present invention. The insertion of genes encoding one or several peptides or proteins which are recognised as an antigen from an infectious agent by the immune system of a mammal is especially preferred. Alternatively, the method of the present invention may be performed using heterologous genes encoding at least one molecule interfering with the replication of an infectious agent or an antibody providing protection against an infectious agent. The heterologous sequences may contain sequences encoding an immune modulator, a cytokine, an immunenhancer and/or an anti-inflammatory compound.

The method of the present invention may be performed using a DNA for cloning into a BAC that has any size. However, specific advantages over the known methods to prepare subcloned DNA from viral are obtained, if large sequences are used. The DNA cloned into the BAC may thus comprise a length of at least 5 Kb, whe-

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rein DNA with a size of at least 15, 25 or 30 Kb is specifically preferred.

According to specifically preferred embodiments of the present invention methods are provided, wherein the BAC comprises a sequence controlling the transcription of the DNA cloned into the BAC. This will allow transcription of the viral RNA and thus enable expression of the virus. Any sequence controlling transcription known in the art may be used for this purpose, including sequences driving the expression of genes derived from DNA or RNA genomes. The use of the immediately early (IE) promoter of cytomegalovirus (CMV) is preferred.

The DNA cloned into the BAC may also be flanked at the 3'-end by a poly(A)tail. The nucleic acid may comprise termination and/or polyadenylation sequences of bovine growth hormone (BGH). Additionally or alternatively, the nucleic acids may comprise sequences encoding a ribozyme, for example the ribozyme of the hepatitis  $\delta$  virus (HDV).

Additional advantages may be achieved if at least one of the genes of the virus has been modified by substituting, deleting or adding nucleotides. For example the gene controlling tropism of the virus may be modified to obtain viruses with altered tropism. Alternatively, the gene controlling tropism of the virus has been substituted with the respective gene of another virus. The modification is preferably performed in the S, M and/or N genes of the virus.

In a preferred embodiment of the present invention a method is provided, wherein the DNA cloned into the BAC is capable of being transcribed into RNA which RNA can be assembled to an virion. The virion may be an infectious, attenuated, replication defective or inactivated virus.

Any RNA virus may be used in the methods of the invention. The virus can for example be a picornavirus, flavivirus, togavirus,

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coronavirus, toroviruses, arteriviruses, calcivirus, rhabdovirus, paramixovirus, filovirus, bornavirus, orthomyxovirus, bunyavirus, arenavirus or reovirus. The use of viruses naturally having a plus strand genome is preferred.

Additionally, the present invention provides methods of preparing a viral RNA or a virion comprising steps, wherein a DNA is prepared according to one of above methods, the DNA is expressed in a suitable host cell and the viral RNA or the virion is isolated from that host cell. Any of methods for isolating viruses from the cell culture known in the art may be used. Alternatively, methods of preparing a viral RNA or a virion are disclosed, wherein the DNA of the present invention is transcribed or translated using chemicals, biological reagents and/or cell extracts and the viral RNA or the virion is subsequently isolated. For certain embodiments, the virus may subsequently be inactivated or killed.

The invention also provides methods for preparing a pharmaceutical composition comprising steps, wherein a DNA, a viral RNA or a virion is prepared according to one of the above methods and is subsequently mixed with a pharmaceutically acceptable adjuvans and/or carrier. A large number of adjuvans and carriers and diluents are known in the prior art and may be used in accordance with the present invention. The pharmaceutical is preferably a vaccine for protecting humans or animals against an infectious disease. The pharmaceutical can advantageously also be used for gene therapy.

The present invention further provides for the first time a DNA comprising sequences derived from the genomic RNA (gRNA) of a coronavirus which sequences have a homology of at least 60% to the natural sequence of the coronavirus and code for an RNA dependent RNA polymerase and at least one structural or non-structural protein, wherein a fragment of said DNA is capable of being transcribed into RNA which can be assembled to a virion.

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According to the present invention the term "sequence derived from a coronavirus" is used to refer to a nucleic acid sequence which has a homology of at least 60%, preferably 75% and more preferably 85 or 95%, to a natural sequence of a coronavirus. Sequence homology can be determined using the Clustal computer program available from the European Bioinformatics Institute (EBI).

The term "coronavirus" is used according to the present invention to refer to a group of viruses having a single molecule of linear, positive sense, ssRNA of 25 to 33 Kb. These viruses usually contain 7 to 10 structural genes, i.e. genes encoding proteins that determine the viral structure. These genes are typically arranged in the viral genome in the order of 5' replicase-(hemagglutinin-esterase)-spike-envelope-membrane-nucleoprotein-3'. Additionally the viral genome may comprise a number of non-structural genes which encode a nested set of mRNAs with a common 3' end and are largely non-essential.

The term "capable of being transcribed into RNA which can be assembled into a virion" is used to characterize a DNA sequence, which - once introduced into a suitable host cell - will be transcribed into RNA and generate virions. The virions are preferably infectious viruses, but may also be inactivated, attenuated or replication defective viruses comprising said RNA. Preferably all the information necessary for expression of the virion is encoded by the DNA sequence of the present invention.

The nucleic acids of the present invention may further comprise a sequence encoding one or several heterologous genes of interest. According to the present invention a gene is characterized as a "heterologous gene" if it is not derived from the coronavirus which was used as a source for the genes encoding the RNA dependent RNA polymerase and the structural or non-structural protein. A "heterologous gene" thus also refers to genes derived from one type of coronavirus and expressed in a vector comprising sequences derived from another type of coronavirus. Any

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heterologous gene of interest can be inserted into the nucleic acids of the present invention. The insertion of genes encoding peptides or proteins which are recognised as an antigen from an infectious agent by the immune system of a mammal is especially preferred. The heterologous gene may thus encode at least one antigen suitable for inducing an immune response against an infectious agent, at least one molecule interfering with the replication of an infectious agent or an antibody providing protection against an infectious agent. Alternatively or additionally, the heterologous gene may encode an immune modulator, a cytokine, an immunenhancer or an anti-inflammatory compound.

The fragment of the DNA according to the present invention which is transcribed into RNA preferably has a size of at least 25 Kb. Fragments with a size of at least 30 Kb are especially preferred.

According to a preferred embodiment of the present invention the DNA further comprises sequences derived from a coronavirus coding for several or all except one of the structural or non-structural proteins of a coronavirus. Alternatively, the DNA of the present invention further comprises sequences coding for all of the structural or non-structural proteins of a coronavirus.

According to a further embodiment, the nucleic acids of the present invention comprise a sequence controlling the transcription of a sequence derived from a coronavirus gRNA. Any sequence controlling transcription known in the art may be used for this purpose, including sequences driving the expression of genes derived from DNA or RNA genomes. The use of the immediately early (IE) promoter of cytomegalovirus (CMV) is preferred.

The nucleic acid according to the present invention may also be flanked at the 3'-end by a poly(A)tail. The nucleic acid may comprise termination and/or polyadenylation sequences of bovine growth hormone (BGH). Additionally or alternatively, the nucleic acids may comprise sequences encoding a ribozyme, for example



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the ribozyme of the hepatitis  $\delta$  virus (HDV).

The nucleic acids of the present invention may comprise sequences derived from any coronavirus, for example derived from an isolate of the porcine transmissible gastroenteritis virus (TGEV), murine hepatitis virus (MHV), infectious bronchitis virus (IBV), bovine coronavirus (BoCV), canine coronavirus (CCoV), feline coronavirus (FCoV) or human coronavirus. According to a preferred embodiment the nucleic acid is derived from a transmissible gastroenteritis virus.

According to a further embodiment of the present invention, the DNAs of the present invention are part of a plasmid, preferably part of a bacterial artificial chromosome (BAC).

The present invention further provides host cells comprising respective nucleic acids or vectors. The host cells may be eucaryotes or procaryotes. Alternatively, the present invention provides virions comprising a nucleic acid according to the present invention. Respective virions may for example be isolated from cell cultures transfected or infected with the nucleic acids of the present invention.

According to a further embodiment, the present invention provides methods for producing a virion or a viral RNA comprising steps, wherein a DNA of the present invention is introduced into a host cell, host cells containing the DNA are cultivated under conditions allowing the expression thereof and the virion or viral RNA is recovered. Additionally, methods for producing a virion or a viral RNA are provided, wherein a DNA of the present invention is mixed in vitro with chemicals, biological reagents and/or cell extracts under conditions allowing the expression of the DNA and the virion or viral RNA is recovered. The present invention also encompasses the virions and viral RNAs obtainable by either of the above methods. RNAs and virions carrying a heterologous gene are preferred. The viruses so obtained may have the form of an infectious, attenuated, replication defecti-

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ve or inactivated virus.

The virus may comprise modified genes, for example a modified S, N or M gene. In a specific embodiment of the present invention the modification of the S, N or M gene gives rise to an attenuated virus or a virus with altered tropism.

According to a further embodiment the invention provides a pharmaceutical preparation comprising nucleic acids, host cells or virions according to the present invention. According to a preferred embodiment the pharmaceutical preparation is a vaccine capable of protecting an animal against diseases caused by an infectious agent. The vaccine may for example comprise sequences of at least one antigen suitable for inducing an immune response against the infectious agent or an antibody providing protection against said infectious agent. The vaccine may comprise a DNA expressing at least one molecule interfering with the replication of the infectious agent. Alternatively the vaccine may comprise a vector expressing at least one antigen capable of inducing a systemic immune response and/or an immune response in mucous membranes against different infectious agents that propagate in respiratory, intestinal mucous membranes or in other tissues. The vaccine may also be a multivalent vaccine capable of protecting an animal against the infection caused by more than one infectious agent, that comprises more than one nucleic acid of the present invention each of which expresses an antigen capable of inducing an immune response against each of said infectious agents, or antibodies that provide protection against each one of said infectious agents or other molecules that interfere with the replication of any infectious agent.

The vaccines of the present invention may further comprise any of the pharmaceutically acceptable carriers or diluents known in the state of the art.

The present invention further provides methods for preparing a DNA of the present invention comprising steps, wherein an in-

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terfering defective genome derived from a coronavirus is cloned under the expression of a promotor into a BAC vector and the deletions within the defective genome are re-inserted. The method may further comprise steps, wherein toxic sequences within the viral genome are identified before re-insertion into the remaining genomic DNA. Preferably, the toxic sequences within the viral genome are the last sequences to be re-inserted before completing the genome. According to the present invention this method is suitable to yield infectious clones of coronaviruses which are stable in bacteria for at least 80 generations and thus provides a very efficient cloning vector.

The present invention provides the development of infective clones of cDNA derived from coronavirus (Almazan et al., 2000), as well as vectors constructed from said infective clones that also include heterologous nucleic acid sequences inserted into said clones. The infective clones and vectors provided by this invention have numerous applications in both basic and applied research, as well as a high cloning capacity, and can be designed in such a way that their species specificity and tropism can be easily controlled.

This patent describes the development of a method that makes it possible to obtain, for the first time in the history of coronavirus, a full-length infective cDNA clone that codes the genome of a coronavirus (Almazan et al., 2000).

A new vector or system of expression of heterologous nucleic acids based on a coronavirus generated from an infective cDNA clone that codes the genomic RNA (gRNA) of a coronavirus has been developed. In one particular realization of this invention, the coronavirus is the porcine transmissible gastroenteritis virus (TGEV).

The new system of expression can be used in basic or applied research, for example, to obtain products of interest (proteins, enzymes, antibodies, etc.), as a vaccinal vector, or in gene

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therapy in both humans and animals. The infective coronavirus obtained from the infective cDNA clone can be manipulated by conventional genetic engineering techniques so that new genes can be introduced into the genome of the coronavirus, and so that these genes can be expressed in a tissue- and species-specific manner to induce an immune response or for gene therapy. In addition, the expression has been optimized by the selection of new transcription-regulating sequences (TRS) that make it possible to increase the levels of expression more than a hundredfold.

The vectors derived from coronavirus, particularly TGEV, present several advantages for the induction of immunity in mucous membranes with respect to other systems of expression that do not replicate in them: (i) TGEV infects intestinal and respiratory mucous membranes (Enjuanes and Van der Zeijst, 1995), that is, the best sites for induction of secretory immunity; (ii) its tropism can be controlled by modifying the S (spike) gene (Bal- lesteros et al., 1997); (iii) there are nonpathogenic strains for the development of systems of expression that depend on complementing virus (Sánchez et al., 1992); and (iv) coronaviruses are cytoplasmic RNA viruses that replicate without passing through an intermediate DNA stage (Lai and Cavanagh, 1997), making its integration into the cellular chromosome practically impossible.

The procedure that has made it possible to recover an infective coronavirus from a cDNA that codes the gRNA of a coronavirus includes the following strategies:

- (i) expression of the RNA of the coronavirus under the control of an appropriate promoter;
- (ii) cloning of the genome of the coronavirus in bacterial artificial chromosomes (BACs);
- (iii) identification of the sequences of cDNA of the coronavirus that are directly or indirectly toxic to bacteria;
- (iv) identification of the precise order of joining of the

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- components of the cDNA that codes an infective RNA of coronavirus (promoters, transcription-termination sequences, polyadenylation sequences, ribozymes, etc.); and
- (v) identification of a group of technologies and processes (conditions for the growth of BACs, modifications to the purification process of BAC DNA, transformation techniques, etc.) that, in combination, allow the efficient rescue of an infective coronavirus of a cDNA.

The promoter plays an important role in increasing the expression of viral RNA in the nucleus, where it is synthesized, to be transported to the cytoplasm later on.

The use of BACs constitutes one of the key points of the procedure of the invention. As is known, cloning of eukaryotic sequences in bacterial plasmids is often impossible due to the toxicity of the exogenous sequences for bacteria. In these cases, the bacteria usually eliminate toxicity by modifying the introduced sequences. Nevertheless, in the strategy followed in this case, to avoid the possible toxicity of these viral sequences, the necessary clonings were carried out to obtain a complete cDNA of the coronavirus in BACs. These plasmids appear in only one copy or a maximum of two per cell, considerably limiting their toxicity and reducing the possibilities of interplasmid recombination.

Through the identification of the bacteriotoxic cDNA sequences of the coronavirus, the construction of the cDNA that codes the complete genome of a coronavirus can be completed, with the exception of the toxic sequences, which are added in the last step of construction of the complete genome, that is, just before transfection in eukaryotic cells, avoiding their modification by the bacteria.

One object of the present invention therefore consists in an infective double-chain cDNA clone that codes the gRNA of a coro-

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navirus, as well as the procedure for obtaining it.

An additional object of this invention consists in a set of recombinant viral vectors that comprises said infective clone and sequences of heterologous nucleic acids inserted into said infective clone.

An additional object of this invention consists in a method for producing a recombinant coronavirus that comprises the introduction of said infective clone into a host cell, the culture of the transformed cell in conditions that allow the replication of the infective clone and production of the recombinant coronavirus, and recovering the recombinant coronavirus from the culture.

Another object of this invention consists in a method for producing a modified recombinant coronavirus that comprises introducing the recombinant viral vector into a host cell; cultivating it in conditions that allow the viral vector to replicate and the modified recombinant coronavirus to be produced, and recovering the modified recombinant coronavirus from the culture.

Another object of this invention consists in a method for producing a product of interest that comprises cultivating a host cell that contains said recombinant viral vector in conditions that allow the expression of the sequence of heterologous DNA.

Cells containing the aforementioned infective clones or recombinant viral vector constitute another object of the present invention.

Another object of this invention consists in a set of vaccines that protect animals against infections caused by infectious agents. These vaccines comprise infective vectors that express at least one antigen adequate for inducing an immune response against each infective agent, or at least one antibody that provides protection against said infective agent, along with a pharmaceutically acceptable excipient. The vaccines can be mono-

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or multivalent, depending on whether the vectors express one or more antigens capable of inducing an immune response to one or more infectious agents, or, alternatively, one or more antibodies that provide protection against one or more infectious agents.

Another object provided by this invention comprises a method of animal immunization that consists in the administration of said vaccine.

The invention provides a cDNA clone that codes the infective RNA of a coronavirus, henceforth the infective clone of the invention, which comprises: (1) a copy of the complementary DNA (cDNA) to the infective genomic RNA (gRNA) of a coronavirus or the viral RNA itself; and (2) an expression module for an additional gene, which includes optimized transcription-promoting sequences.

In one particular realization of this invention, the coronavirus is a TGEV isolate, in particular, the PUR46-MAD isolate (Sánchez et al., 1990), modified by the replacement of the S gene of this virus by the S gene of the clone C11 TGEV isolate or the S gene of a canine or human coronavirus.

The transcription-promoting sequence, or promoter, is an RNA sequence located at the 5'-terminal end of each messenger RNA (mRNA) of coronavirus, to which the viral polymerase RNA binds to begin the transcription of the messenger RNA (mRNA). In a particular and preferred embodiment the viral genome is expressed from a cDNA using the IE promoter of CMV, due to the high level of expression obtained using this promoter (Dubensky et al., 1996), and to previous results obtained in our laboratory that indicated that large defective genomes (9.7 kb and 15 kb) derived from the TGEV coronavirus expressed RNAs that did not undergo splicing during their transport from the nucleus, where they are synthesized, to the cytoplasm.

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The infective clone of the invention also contains a transcription termination sequence and a polyadenylation signal such as that coming from the BGH gene. These termination sequences have to be placed at the 3'-end of the poly(A) tail. In one particular realization, the infective clone of the invention contains a poly(A) tail of 24 residues of A and the termination and polyadenylation sequences of the BGH separated from the poly(A) tail by the sequence of the HDV ribozyme.

The plasmid into which the infective cDNA of the virus has been inserted is a DNA molecule that possesses a replication origin, and is therefore potentially capable of replicating in a suitable cell. The plasmid used is a replicon adequate for maintaining and amplifying the infective clone of the invention in an adequate host cell such as a bacterium, for example, *Escherichia coli*. The replicon generally carries a gene of resistance to antibiotics that allows the selection of the cells that carry it (for example, cat).

In Example 1, the construction of an infective clone of TGEV under the control of the IE promoter of CMV is described. The 3'-end of the cDNA appears flanked by a 24 nt poly(A) sequence, the HDV ribozyme, and the transcription termination sequence of the BGH.

The procedure for obtaining the infective clone of the invention comprises constructing the full-length cDNA from the gRNA of a coronavirus and joining the transcription-regulating elements.

The cDNA that codes the infective gRNA of a coronavirus was obtained from a DI genome derived from a coronavirus cloned as a cDNA under the control of an appropriate promoter in a BAC, for the purpose of increasing the cDNA's stability. Then the bacteriotoxic sequences were identified and, for the purpose of eliminating that toxicity, said toxic sequences were removed and inserted at the end of the construction of the complete genome, just before transfection in eukaryotic cells. The viral progeny



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can be reconstituted by means of transfection of the BAC plasmid that contains the coronavirus genome in eukaryotic cells that support viral replication.

The transcription-regulating elements are joined by means of conventional techniques (Maniatis et al., 1989).

The infective clone of the invention can be manipulated by conventional genetic engineering techniques to insert at least one sequence of a heterologous nucleic acid that codes a determined activity, under the control of the promoter that is present in the infective clone and of the regulating sequences contained in the resulting expression vector.

The infective clone of the invention presents numerous applications; for example, it can be used both in basic research, for example, to study the mechanism of replication and transcription of coronaviruses, and in applied research, for example, in the development of efficient systems of expression of products of interest (proteins, enzymes, antibodies, etc.).

Appropriate cells can be transformed from the infective cDNA clone of the invention, and the virions obtained containing the complete genome of the coronavirus can be recovered. Therefore, the invention moreover provides a method for producing a recombinant coronavirus that comprises the introduction of an infective cDNA of the invention into a host cell, the culture of said cell under conditions that allow the expression and replication of the infective clone and the recovery of the virions obtained from the recombinant coronavirus, which contain the infective genome of the coronavirus. The infective clone of the invention can be introduced into the host cell in various ways, for example by transfection of the host cell with an RNA transcribed in vitro from an infective clone of the invention, or by infecting the host cell with the infective cDNA clone of the invention. Said host cells that contain the infective clone of the invention constitute an additional object of the present inven-

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tion.

The invention also provides a set of recombinant viral vectors derived from an infective clone of the invention, henceforth viral vectors of the invention. The viral vectors of the invention comprise an infective cDNA clone of the invention modified to contain a heterologous nucleic acid inserted into said infective clone under conditions that allow said heterologous nucleic acid to be expressed.

The term "nucleic acid," as it is used in this description, includes genes or gene fragments as well as, in general, any molecule of DNA or RNA.

In the sense used in this description, the term "heterologous" applied to a nucleic acid refers to a nucleic acid sequence that is not normally present in the vector used to introduce the heterologous nucleic acid into a host cell.

The heterologous nucleic acid that can contain the viral vector of the invention can be a gene or fragment that codes a protein, a peptide, an epitope, or any gene product of interest (such as antibodies, enzymes, etc.). The heterologous nucleic acid can be inserted into the infective clone of the invention by means of conventional genetic engineering techniques in any appropriate region of the cDNA, for example, after ORF 1b or between genes N and 7, following the initiator codon (AUG), and in reading frame with that gene; or, alternatively, in the zones corresponding to other ORFs. In the construction of the viral vector of the invention, it is essential that the insertion of the heterologous nucleic acid not interfere with any of the basic viral functions.

The viral vector of the invention can express one or more activities. In this latter case, the viral vector will include as many sequences of heterologous nucleic acid as activities to be expressed, preceded by one or several promoters, or by a promo-

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ter and various ribosome recognition sites (IRES, internal ribosome entry sites), or by various promoters and one ribosome recognition site.

Therefore, the invention provides a method for producing a product of interest that comprises cultivating a host cell that contains a viral vector of the invention under conditions that allow the heterologous nucleic acid to be expressed and the product of interest to be recovered. Said host cells that contain the viral vector of the invention constitute an additional object of the present invention.

The viral vector of the invention can be designed so that its species specificity and tropism can be easily controlled. Due to these characteristics, a very interesting application of the viral vectors of the invention is their use in gene therapy as a vector of the gene of interest, or as a vaccinal vector to induce immune responses against different pathogens.

The invention furthermore provides vaccines, capable of protecting an animal against the infection caused by an infectious agent, that comprise (i) at least one viral vector of the invention that expresses at least one antigen suitable for inducing an immune response against said infectious agent, or an antibody that provides protection against said infectious agent, along with, optionally, (ii) a pharmaceutically acceptable excipient.

In the sense used in this description, "inducing protection" should be understood as the immune response of the receiving organism (animal to be immunized) induced by the viral vector of the invention, through suitable mechanisms such as that induced by substances that potentiate cellular response (interleukins, interferons, etc.), cellular necrosis factors, and similar substances that protect the animal from infections caused by infectious agents.

Included under the term "animal" are all animals of any species,

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preferably mammals, including man.

The term "infectious agent" in the sense used in this description includes any viral, bacterial, fungal, parasitic, or other infective agent that can infect an animal and cause it a pathology.

In one particular realization, the vaccine provided by this invention comprises at least one viral vector of the invention that expresses at least one antigen capable of inducing a systemic immune response and/or an immune response in mucous membranes against different infectious agents that propagate in respiratory or intestinal mucous membranes. The vectors of the invention are quite suitable to induce immunity in mucous membranes as well as lactogenic immunity, which is of special interest in protecting newborns against intestinal tract infections.

In another particular realization, the vaccine provided by this invention comprises at least one viral vector of the invention that expresses at least one gene that codes for the light and heavy chains of an antibody of any isotype (for example, IgG<sub>1</sub>, IgA, etc.) that protects against an infectious agent.

Species specificity can be controlled so that the viral vector may express the S protein of the envelope of a coronavirus that infects the desired species (man, dog, cat, pig, etc.), suitable to be recognized by the cellular receptors of the corresponding species.

The vaccines provided by this invention can be monovalent or multivalent, depending on whether the viral vectors of the invention express one or more antigens capable of inducing an immune response to one or more infectious agents, or one or more antibodies that provide protection against one or more infectious agents.

In a particular realization of this invention, monovalent vacci-

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nes capable of protecting man, pigs, dogs and cats against different infectious human, porcine, canine, and feline agents are provided, and tropism is controlled by expressing the S glycoprotein of the coronavirus with the desired species specificity.

The monovalent vaccines against porcine infectious agents can contain a vector that expresses an antigen selected from the group consisting essentially of antigens of the following porcine pathogens: *Actinobacillus pleuropneumoniae*, *Actinobacillus suis*, *Haemophilus parasuis*, porcine parvovirus, *Leptospira*, *Escherichia coli*, *Erysipelotrix rhusiopathiae*, *Pasteurella multocida*, *Bordetella bronchiseptica*, *Clostridium* sp., *Serpulina hydrosentariae*, *Mycoplasma hyopneumoniae*, porcine epidemic diarrhea virus (PEDV), porcine respiratory coronavirus, rotavirus, or against the pathogens that cause porcine respiratory and reproductive syndrome, Aujeszky's disease (pseudorabies), swine influenza, or transmissible gastroenteritis, and the etiological agent of atrophic rhinitis and proliferative ileitis. The monovalent vaccines against canine infectious agents can contain an expression vector that expresses an antigen selected from the group essentially consisting of antigens of the following canine pathogens: canine herpes viruses, types 1 and 2 canine adenovirus, types 1 and 2 canine parvovirus, canine reovirus, canine rotavirus, canine coronavirus, canine parainfluenza virus, canine influenza virus, distemper virus, rabies virus, retrovirus, and canine calicivirus.

The monovalent vaccines against feline infectious agents can contain an expression vector that expresses an antigen selected from the group essentially consisting of antigens of the following feline pathogens: cat calicivirus, feline immunodeficiency virus, feline herpes viruses, feline panleukopenia virus, feline reovirus, feline rotavirus, feline coronavirus, cat infectious peritonitis virus, rabies virus, feline *Chlamydia psittaci*, and feline leukemia virus.

The vectors can express an antibody that provides protection

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against an infectious agent, for example, a porcine, canine or feline infectious agent such as those cited above. In one particular realization, the vector expresses the recombinant monoclonal antibody identified as 6A.C3, which neutralizes TGEV, expressed with isotypes IgG<sub>1</sub> or IgA, in which the constant part of the immunoglobulin is of porcine origin, or neutralizing antibodies for human and porcine rotaviruses.

As the excipient, a diluent such as physiological saline or other similar saline solutions can be used. Likewise, these vaccines can also contain an adjuvant from those usually used in the formulation of both aqueous vaccines, such as aluminum hydroxide, QuilA, suspensions of alumina gels and the like, and oily vaccines based on mineral oils, glycerides, fatty acid derivatives, and their mixtures.

The vaccines of the present invention can also contain cell-response-potentiating (CRP) substances, that is, substances that potentiate subpopulations of helper T-cells (Th<sub>1</sub> and Th<sub>2</sub>) such as interleukin-1 (IL-1), IL-2, IL-4, IL-5, IL-6, IL-12, gamma-IFN (gamma-interferon), cellular necrosis factor, and similar substances that could theoretically provoke cellular immunity in vaccinated animals. These CRP substances could be used in vaccine formulations with aqueous or oily adjuvants. Another type of adjuvants that modulate and immunostimulate cellular response can also be used, such as MDP (muramyl dipeptide), ISCOM (Immunostimulant Complex), or liposomes.

The invention provides multivalent vaccines capable of preventing and protecting animals from infections caused by different infectious agents. These multivalent vaccines can be prepared from viral vectors of the invention into which the different sequences that code the corresponding antigens have been inserted in the same recombinant vector, or by constructing independent recombinant vectors that would later be mixed for joint inoculation. Therefore, these multivalent vaccines comprise a viral vector that contains more than one sequence of heterolo-

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gous nucleic acids that code for more than one antigen or, alternatively, different viral vectors, each of which expresses at least one different antigen.

Analogously, multivalent vaccines that comprise multivalent vectors can be prepared using sequences that code antibodies that protect against infectious agents, instead of sequences that code the antigens.

In one particular realization of this invention, vaccines capable of immunizing humans, pigs, dogs, and cats against different porcine, canine and feline infectious agents, respectively, are provided. For this, the viral vectors contained in the vaccine must express different antigens of the human, porcine, canine or feline pathogens mentioned above or others.

The vaccines of this invention can be presented in liquid or lyophilized form and can be prepared by suspending the recombinant systems in the excipient. If said systems were in lyophilized form, the excipient itself could be the reconstituting substance.

Alternatively, the vaccines provided by this invention can be used in combination with other conventional vaccines, either forming part of them or as a diluent or lyophilized fraction to be diluted with other conventional or recombinant vaccines.

The vaccines provided by this invention can be administered to the animal orally, nasally, subcutaneously, intradermally, intraperitoneally, intramuscularly, or by aerosol.

The invention also provides a method for the immunization of animals, in particular pigs, dogs and cats, against one or various infectious agents simultaneously, that comprises the oral, nasal, subcutaneous, intradermal, intraperitoneal, intramuscular, or aerosol administration (or combinations thereof) of a vaccine that contains an immunologically efficacious quantity of

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a recombinant system provided by this invention.

In addition, the invention also provides a method for protecting newborn animals against infectious agents that infect said animals, consisting in the oral, nasal, subcutaneous, intradermal, intraperitoneal, intramuscular, or aerosol administration (or combinations thereof) of a vaccine of those provided by this invention to mothers before or during the gestation period, or to their offspring.

The invention is illustrated by the following examples, which describe in detail the obtainment of infective clones and the construction of the viral vectors of the invention. These examples should not be considered as limiting the scope of the invention, but as illustrating it. In said example, the transformation and growth of bacteria, DNA purification, sequence analysis, and the assay to evaluate the stability of the plasmids were carried out according to the methodology described below.

#### Transformation of bacteria

All of the plasmids were electroporated in the *E. coli* DH10B strain (Gibco BRL), introducing slight modifications to previously described protocols (Shizuya et al., 1992). For each transformation, 2  $\mu$ L of the ligation and 50  $\mu$ L of competent bacteria were mixed in 0.2-cm dishes (BioRad) and electroporated at 200  $\Omega$ , 2.5 kV, and 25  $\mu$ F. Then, 1 mL of SOC medium (Maniatis et al., 1989) was added at each transformation, the cells were incubated at 37°C for 45 min, and finally, the recombinant colonies were detected on plates of LB SOC media (Maniatis et al., 1989) with 12.5  $\mu$ g/mL of chloramphenicol.

#### Growth conditions of the bacteria

The bacteria containing the original plasmids, in which the incomplete genome of TGEV was cloned (Figure 3), were grown at 37°C, showing normal growth kinetics. On the other hand, the BAC that contained the complete cDNA was grown at 30°C for the pur-



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pose of minimizing instability as much as possible. Even so, the size of the colonies was reduced and incubation periods of up to 24 h were necessary to achieve normal colony sizes.

#### Purification of DNA

The protocol described by Woo (Woo et al., 1994) was followed, with slight modifications. From a single colony, 4 L of LB were inoculated with chloramphenicol (12.5 µg/ml). After an incubation period of 18 h at 30°C, the bacteria were collected by centrifugation at 6,000 G, and the plasmid was purified using the Qiagen Plasmid Maxipreparations kit according to the manufacturer's recommendations. By means of this procedure, it was observed that the plasmid DNA obtained was contaminated with bacterial DNA. To eliminate the contaminating bacterial DNA, the plasmidic DNA was purified by means of centrifugation at 55,000 rpm for 16 h on a CsCl gradient. The yield obtained was between 15 and 30 µg/L, depending on the size of the plasmid.

#### Sequence Analysis

The DNA was sequenced in an automatic sequencer (373 DNA Sequencer, Applied Biosystems) using dideoxynucleotides marked with fluorochromes and a temperature-resistant polymerase (Perkin Elmer). The reagents were obtained by way of a kit (ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit) from the Applied Biosystems company. The thermocycler used to perform the sequencing reactions was a "GeneAmpPCR System 9600" (Perkin Elmer).

The joining of the sequences and their comparison with the consensus sequence of the TGEV were carried out using the SeqMan II and Align (DNASTAR) programs, respectively. No differences in relation to the consent sequence were detected.

#### Stability of the plasmids

From the original glycerolates, the bacteria that contained recombinant pBeloBAC11 plasmids were grown in 20 mL of LB with chloramphenicol (12.5 µg/mL) for 16 h at 30°C and 37°C. This

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material was considered passage 0. The bacteria were diluted  $10^6$  times and grown at 30°C and 37°C for 16 h. Serial passages were realized during eight consecutive days (each passage represents approximately 20 generations). The plasmid DNA was purified by Miniprep at passages 0 and 8 (160 generations) and analyzed with restriction endonucleases. The two plasmids that contained part of the genome of TGEV were highly stable, whereas the plasmid that contained the complete genome of TGEV showed a certain instability after 40 generations (at this point approximately 80% of the DNA presented the correct restriction pattern).

#### Example 1

##### CONSTRUCTION OF A RECOMBINANT VECTOR

##### BASED ON A CLONE OF INFECTIVE cDNA DERIVED FROM TGEV

#### 1.1 Generation of an infective cDNA of TGEV

For the purpose of obtaining a cDNA that coded for the complete TGEV genome, we originally started with a cDNA that coded for the defective DI-C genome (Méndez et al., 1996). This cDNA, with an approximate length of one third of the TGEV genome, was cloned in the low-copy pACNR1180 plasmid (Ruggli et al., 1996) and its sequence was determined. The cDNA that coded the defective genome was efficiently rescued (replicated and packaged) with the help of a complementing virus (Méndez et al., 1996; Izeta et al., 1999).

The DI-C genome presents three deletions ( $\Delta 1$ ,  $\Delta 2$ , and  $\Delta 3$ ) of approximately 10, 1 and 8 kilobases (kb), at ORFs 1a, 1b, and between genes S and 7, respectively (see Figure 1).

The strategy followed to complete the sequence of a cDNA that would code for an infective TGEV genome was to incorporate, step by step, the sequences deleted in the DI-C genome, analyzing the bacteriotoxicity of the new generated constructions. This aspect is very important, since it is widely documented in the scientific literature that recombinant plasmids presenting cDNAs of RNA virus generally grew poorly and were unstable (Boyer and Haenni, 1994; Rice et al., 1989; Mandl et al., 1997).

The first deletion to be completed was deletion  $\Delta 2$ , of 1 kb, of

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ORF 1b, yielding a stable recombinant plasmid. The sequence that lacked ORF 1a was introduced by cloning cDNA fragments A, B, C, and D (Figure 1) (Almazan et al., 2000) in such a way that all the information required for the gene of the replicase would be complete. The recombinant plasmid obtained was unstable in the bacteria, generating new plasmids that had incorporated additions and deletions in fragment B (Almazan et al., 2000). Interestingly, the elimination of a 5,198 bp *Cla*I-*Cla*I restriction fragment that encompassed the region of the genome comprised between nucleotides 4,417 and 9,615 (Penzes et al., 1999) yielded a relatively stable plasmid in the *E. coli* DH10B strain. Later, the sequence of deletion  $\Delta 3$  was added by cloning all the genetic information for the structural and nonstructural proteins of the 3'-end of the TGEV genome (Figure 1).

For the purpose of incrementing the stability of the TGEV cDNA, it was decided that it would be subcloned in BAC using the pBel-oBAC11 plasmid (Kim et al., 1992) (see Figure 2). The pBel-oBAC11 plasmid was a generous gift from H. Shizuya and M. Simon (California Institute of Technology). The plasmid, 7,507 bp in size, includes the replication origin of the F factor from *parB*, *parC*, *E. coli* (*oriS*) and the genes necessary to keep a single copy of the plasmid per cell (*parA*, and *repE*). The plasmid also presents the gene of resistance to chloramphenicol (*cat*) as a selection marker. The cDNA was cloned under the control of the IE promoter of CMV, due to the high level of expression obtained using this promoter (Dubensky et al., 1996) and to previous results obtained in our laboratory, indicating that large (9.7 kb and 15 kb) defective genomes derived from TGEV expressed RNAs that did not undergo splicing during transport from the nucleus, where they are synthesized, to the cytoplasm (Izeta et al., 1999; Penzes et al., 1999; Almazan et al., 2000). The generated TGEV cDNA (pBAC-TcDNA- $\Delta$ *Cla*I) contained the information for the genes of the replicase, with the exception of the deleted 5,198 bp *Cla*I fragment, and all the information of the structural and nonstructural genes. The 3'-end of the cDNA appears flanked by a 24 nt polyA sequence, the HDV ribozyme, and the transcription termination sequence of BGH (Izeta et al., 1999). On the other

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hand, the *Cla*I fragment necessary to generate a complete genome of TGEV was cloned in BAC, generating the plasmid pBAC-B+C+D5', which contained the region of the TGEV genome between 4,310 and 9,758 (see Figure 3). Both plasmids were grown in the *E. coli* DH10B strain and sequenced in their entirety. The sequence obtained was identical to the consent sequence of the PUR46-MAD isolate of TGEV provided at the end of this document (SEQ ID NO:1), with the exception of two replacements in the positions of nucleotides 6,752 (A => G, silent) and 18,997 (T => C, silent), and the changes in the S gene of the PUR46-MAD that has been replaced by the D gene of isolate C11 (these changes are indicated in Figure 4).

Furthermore, for the purpose of generating a cDNA that would code a virulent TGEV, the S gene of the PUR46-MAD isolate, which replicates at high levels in the respiratory tract ( $>10^6$  PFU/g of tissue) and at low levels in the intestinal tract ( $<10^3$  PFU/mL), was completely replaced by the S gene of TGEV clone 11, henceforth C11, which replicates with elevated titers both in the respiratory tract ( $<10^6$  PFU/mL) and in the intestinal tract ( $<10^6$  PFU/mL) (Sánchez et al., 1999). The S gene of C11 presents 14 nucleotides that differ from the S gene of the PUR46-MAD isolate, plus a 6 nt insertion at the 5'-end of the S gene (see Figure 4) (Sánchez et al., 1999). Previous results in our laboratory (Sánchez et al., 1999) showed that mutants generated by directed recombination, in which the S gene of the PUR46-MAD isolate of the TGEV was replaced with the S gene of the C11 intestinal isolate, acquired intestinal tropism and increased virulence, unlike the natural PUR46-MAD isolate of the TGEV that replicates very little or not at all in the intestinal tracts of infected pigs.

A cDNA was constructed from the PUR46-MAD isolate of TGEV with the S gene of the intestinal isolate C11, by means of cloning of the 5,198 bp *Cla*I-*Cla*I fragment, obtained from the pBAC-B+C+D5' plasmid, in the pBAC-TcDNA<sup>-Δ*Cla*I</sup> plasmid, to generate the pBAC--TcDNA<sup>FL</sup> plasmid that contains the cDNA that codes for the complete TGEV genome (Figure 3).

The stability in bacteria of the plasmids used in the construc-

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tion of the clone of infective cDNA (pBAC-TcDNA<sup>-ΔClaI</sup> and pBAC--ClaI<sup>F</sup>), as well as the plasmid that contains the complete cDNA (pBAC-TcDNA<sup>FL</sup>); was analyzed after being grown in *E. coli* for 160 generations. The stability was analyzed by means of digestion with restriction enzymes of the purified DNAs. No deletions or insertions were detected, although the presence of minor changes not detected by the analysis technique used cannot be ruled out in the case of the pBAC-TcDNA<sup>-ΔClaI</sup> plasmid and the pBAC-B+C+D5' plasmid. In the case of the pBAC-TcDNA plasmid, which contains the complete genome of TGEV, a certain instability was detected after 40 generations (at this point approximately 80% of the DNA presented the correct restriction pattern). This slight instability, however, does not represent an obstacle to the rescue of the infective virus, since 20 generations (4 L of culture) of bacterial growth are sufficient to generate a quantity of plasmid DNA that allows the virus to be rescued.

#### 1.2 Rescue of an infective TGEV from a cDNA that codes for the complete genome

ST cells were transfected with the pBAC-TcDNA<sup>FL</sup> plasmid. At 48 h posttransfection, the supernatant of the culture was collected and passed into ST cells six times (see Figure 5). Starting at passage 2, at 14 h postinfection, the cytopathic effect became apparent, extending later, at 20 h postinfection, to practically all of these cells that formed the monolayer (see Figure 6). On the other hand, the titer of rescued virus increased rapidly with the passages, reaching values on the order of 10<sup>8</sup> PFU/mL as of passage 3 (see Figure 7). The experiment was repeated five times, and in all cases, infective virus with similar titers were recovered, whereas, in the case of nontransfected ST cells or ST cells transfected with a similar plasmid, where the ClaI-ClaI fragment was found in the opposite orientation, virus was never recovered.

For the purpose of eliminating the possibility that the virus obtained was the product of contamination, the sequence at positions 6,752 and 18,997 was determined by means of sequencing of cDNA fragments amplified by RT-PCR using the genomic RNA of the

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rescued virus as a template. The analysis of the sequence determined that the nucleotides in positions 6,752 and 18,997 were those present in the cDNA. Furthermore, the rescued virus presented, in the cDNA sequence of the S gene, a restriction site DraIII at position 20,990, as was expected for the S gene of C11 (Figure 8). The presence of these three genetic markers confirmed that the isolated virus came from the cDNA.

In a more in-depth characterization of the virus generated, a comparative analysis was made by immunofluorescence of infected cells with the virus recovered (TcDNA) after transfection with the pBAC-TcDNA<sup>FL</sup> plasmid or cells infected with the PUR46-MAD isolate of the TGEV. For this, specific polyclonal and monoclonal antibodies that recognized both the C11 isolate and the PUR46-MAD isolate, or only the latter, were used (see Figure 10). The results obtained confirmed the antigenicity expected for the new TcDNA virus. The polyclonal antibody specific for TGEV, the expected specific monoclonal of the S protein (ID.B12 and 6A.C3), as well as the specific monoclonal of the M (3B.B3) and N (3B.D8) proteins recognized both the TcDNA and the PUR46-MAD. The data obtained indicated that the virus generated presented the M and N proteins of the PUR46-MAD isolate and the S protein of the C11 isolate, as had been designed in the original cDNA.

### 1.3 In vivo infectivity and virulence

For the purpose of analyzing the in vivo infectivity of the TcDNA virus, a group of five newborn pigs was inoculated with virus cloned from passage 6, and mortality was analyzed. The five inoculated pigs died 3 to 4 days postinoculation, indicating that the TcDNA virus was virulent. In contrast, two pigs inoculated only with the diluent of the virus and maintained in the same conditions did not suffer alterations.

### 1.4 Optimization of the levels of expression by modification of the transcription-regulating sequences

RNA synthesis in coronavirus takes place by means of an RNA-dependent process, in which the mRNAs are transcribed from tem-

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plates with negative polarity. In the TGEV, a conserved consensus sequence, CUAAAC, appears, which is located just in front of the majority of the genes. These sequences represent signals for the transcription of the subgenomic mRNAs. In coronavirus, there are between six and eight types of mRNAs with variable sizes, depending on the type of coronavirus and of the host. The largest corresponds to the genomic RNA, which in turn serves as mRNA for ORFs 1a and 1b. The rest of the mRNAs correspond to subgenomic mRNAs. These RNAs are denominated mRNA 1 to 7, in decreasing size order. On the other hand, some mRNAs that have been discovered after the set of originally described mRNAs have been denominated with the name of the corresponding mRNA, a dash, and a number, e.g., mRNA 2-1. The mRNAs present a coterminal structure in relation to the structure of the genomic RNA. With the exception of the smallest mRNA, the rest are structurally polycistronic, while, in general, only the ORF located closest to 5' is translated.

The efficiency in the expression of a marker gene (GUS) has been studied using different sequences flanking the 5'-terminal of the minimal intergenic (IG) sequence CUAAAC (Figure 11), different sequences flanking the 3'-terminal of the IG sequence (Figure 12), and various insertion sites (Figure 13). The results obtained (Figures 11 to 13) indicated that optimal expression was achieved with a TRS consisting of: (i) the -88 nt flanking the consent sequence for the N gene of TGEV; (ii) the IG sequence; and (iii) the 3'-flanking sequence of the IG sequence of the S gene. Furthermore, in agreement with the results obtained in relationship to the point of insertion of the heterologous gene, the greatest levels of expression were achieved when the heterologous gene was located at the 3'-end of the genome. A TRS such as that described allows the GUS to be expressed at levels between 2 and 8  $\mu\text{g}$  per  $10^6$  cells.

### 1.5 Tissue specificity of the system of expression

Many pathogens enter the host through the mucous membranes. To prevent this type of infections, it is important to develop systems of expression that allow the induction of high levels of

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secretory immunity. This can be achieved fundamentally through the administration of antigens in the lymph nodes associated with the respiratory and intestinal tract. To achieve this goal, and in general to direct the expression of a gene at the tissue of interest, the molecular bases of the tropism of TGEV have been studied. These studies have showed that the tissue specificity of TGEV can be modified by the construction of recombinant viruses containing the S gene of coronavirus with the desired tropism (Ballesteros et al., 1997; Sánchez et al., 1999). This information makes it possible to construct systems of expression based on cDNA genomes of coronavirus with respiratory or intestinal tropism.

#### 1.6 Expression of the viral antigen coded by the ORF5 of PRRSV using infective cDNA

For the purpose of optimizing the levels of expression of heterologous genes, constructions were made from a vector of interchangeable modules flanked by cloning sequences that facilitate the exchange of TRSs and heterologous genes within the vector. The construction, which included ORF 5 of the PRRSV (Porcine respiratory and reproductive syndrome virus) flanked at the 5'-end by the minimal IGS consensus sequence (CUAAAC) preceded by the -88 nts flanking the gene of the viral nucleocapsid (N), and at the 3'-end by restriction site Sall (GTCGAC) and a sequence analogous to that of Kozak (AC)GACC, yielded an optimal expression (about 10  $\mu\text{g}/10^6$  cells). In principle, these levels of expression of the heterologous gene are more than sufficient to induce an immune response. The heterologous gene was inserted into the position previously occupied by genes 3a and 3b of the virus, which are dispensable.

#### 1.7 Induction of an immune response in swine to an antigen expressed with the cDNA derived virus vector

Using the same type of virus vector derived from the cDNA and the TRSs described above, the gene encoding the green fluorescent protein (GFP) was expressed at high levels (20  $\mu\text{g}$  of protein per million of cells in swine testis, ST, cells). The ex-



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pression levels were stable for more than 20 passages in cell culture. Furthermore, a set of swine were immunized with the live virus vector, that was administered by the oral, intranasal and intragastric routes and a strong humoral immune response was detected against both the virus vector and the GFP. Interestingly, no secondary effect was observed in the inoculated animals after the administration of three doses of the virus vector.

1.8 Construction of a safe virus vector that expresses the foreign gene without leading to the generation of an infectious virus.

To design vector for humans, biosafety is a priority. To achieve this goal, three types of safety guards are being engineered in the vector. Two of these are based on the deletion of two virus components, mapping at different positions of the virus genome, essential for the replication of the virus. These components are being provided in *trans* by a packaging cell line. This cell (Baby Hamster Kidney, BHK) expresses the missing TGEV genes encoding essential structural proteins of the virus (the envelope E and the membrane M proteins). The third safety guard is the relocation of the packaging signal of the virus genome, in such a way that the recovery of an infectious virus by recombination is prevented, leading to the generation of a suicide vector that efficiently expresses the heterologous genes but that is unable to propagate even to the closest neighbor cell.

With the design of the new vector for use in humans, we are not producing a new virus that could be propagated within the human species, since this vector can not be transmitted from cell to cell in human beings. The vector is based on a replication defective virus. It can only be grown in the vaccine factory by using packaging cells complementing the deletions of the virus. These safety guards represent novel procedures in the engineering of coronaviruses. The recombinant virus with a new tropism will be replication competent at least in feline cells, since these cells replicate human, porcine, canine and feline coronaviruses.

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## DEPOSITION OF MICROORGANISMS:

The bacterium derived from *Escherichia coli*, carrying the plasmid with the infective clone of the invention, identified as *Escherichia coli* pBAC-TcDNA<sup>FL</sup>, has been deposited with the Spanish Collection of Type Cultures (CECT), Burjassot (Valencia), on November 24<sup>th</sup> 1999, under registration number CECT 5265.

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## Claims .

1. Method of preparing a DNA comprising steps, wherein
  - (a) a DNA comprising a full length copy of the genomic RNA (gRNA) of an RNA virus; or
  - (b) a DNA comprising one or several fragments of a gRNA of an RNA virus, which fragments code for an RNA dependent RNA polymerase and at least one structural or non-structural protein; or
  - (c) a DNA having a homology of at least 60% to the sequences of (a) or (b);is cloned into a bacterial artificial chromosome (BAC).
2. Method according to claim 1, wherein the DNA cloned into the BAC further comprises sequences coding for several or all except one of the structural or non-structural proteins of a virus.
3. Method according to claim 1 or 2, wherein the DNA cloned into the BAC further comprises sequences encoding one or several heterologous gene.
4. Method according to claim 3, wherein the heterologous gene encodes at least one antigen suitable for inducing an immune response against an infectious agent, at least one molecule interfering with the replication of an infectious agent, an antibody providing protection against an infectious agent, an immune modulator, a cytokine, an immoenhancer or an anti-inflammatory compound.
5. Method according to one of claims 1 to 4, wherein the DNA cloned into the BAC has a size of at least 5 Kb.
6. Method according to one of claims 1 to 5, wherein the DNA cloned into the BAC has a size of at least 15 Kb.

7. Method according to one of claims 1 to 6, wherein the DNA cloned into the BAC has a size of at least 25 Kb.
8. Method according to one of claims 1 to 7, wherein the BAC comprises a sequence controlling the transcription of the DNA cloned into the BAC.
9. Method according to one of claims 1 to 8, wherein one of the genes of the virus has been modified by substituting, deleting or adding nucleotides.
10. Method according to claim 9, wherein the gene controlling tropism of the virus has been modified.
11. Method according to one of claims 1 to 10, wherein the gene controlling tropism of the virus has been substituted with the respective gene of another virus.
12. Method according to one of claims 1 to 11, wherein the DNA cloned into the BAC is capable of being transcribed into RNA which RNA can be assembled to a virion.
13. Method according to of claim 12, wherein the virion is an infectious, attenuated, replication defective or inactivated virus.
14. Method according to one of claims 1 to 13, wherein the virus naturally has a plus strand genome.
15. Method according to one of claims 1 to 14, wherein the virus is a picornavirus, flavivirus, togavirus, coronavirus, torovirus, arterivurs, calcivirus, rhabdovirus, paramixovirus, filovirus, bornavirus, orthomyxovirus, bunyavirus, arenavirus or reovirus.
16. Method of preparing a viral RNA comprising steps, wherein a DNA is prepared according to one of claims 1 to 15, the DNA is expressed in a suitable host cell or the DNA is mixed

with chemicals, biological reagents and/or cell extracts under conditions allowing the transcription of the DNA and the viral RNA is isolated.

17. Method of preparing a virion comprising steps, wherein a DNA is prepared according to one of claims 1 to 15, the DNA is expressed in a suitable host cell or the DNA is mixed with chemicals, biological reagents and/or cell extracts under conditions allowing the transcription and translation of the DNA and the virion is isolated.
18. Method according to claim 17, wherein the virion is subsequently inactivated or killed.
19. Method for preparing a pharmaceutical composition comprising steps, wherein a DNA is prepared according to one of claims 1 to 15, a viral RNA is prepared according to claim 16 or a virion is prepared according to claim 17 or 18 and is subsequently mixed with a pharmaceutically acceptable adjuvans or carrier.
20. Method according to claim 19, wherein the pharmaceutical is a vaccine for protecting humans or animals against an infectious disease.
21. Method according to claim 19, wherein the pharmaceutical is used for gene therapy of humans or animals.
22. DNA comprising sequences derived from the genomic RNA (gRNA) of a coronavirus which sequences have a homology of at least 60% to the natural sequence of the virus and code for an RNA dependent RNA polymerase and at least one structural or non-structural protein, wherein a fragment of said DNA is capable of being transcribed into RNA which RNA can be assembled to a virion.
23. DNA according to claim 22, further comprising a sequence

encoding a heterologous gene.

24. DNA according to claim 23, wherein the heterologous gene encodes at least one antigen suitable for inducing an immune response against an infectious agent, at least one molecule interfering with the replication of an infectious agent, an antibody providing protection against an infectious agent, an immune modulator, a cytokine, an immunenhancer or an anti-inflammatory compound.
25. DNA according to claim 22 or 24, wherein said fragment has a size of at least 25 Kb.
26. DNA according to one of claims 22 to 25, which further comprises sequences derived from a coronavirus coding for several or all except one of the structural or non-structural proteins of a virus.
27. DNA according to one of claims 22 to 26, which further comprises sequences derived from a coronavirus coding for all of the structural or non-structural proteins of a coronavirus.
28. DNA according to one of claims 22 to 27, further comprising a sequence controlling the transcription of the viral gRNA.
29. DNA according to one of claims 22 to 28, wherein the sequence controlling transcription of the viral gRNA is the immediately early (IE) promoter of cytomegalovirus (CMV).
30. DNA according to one of claims 22 to 29, wherein the sequence is flanked at the 3'-end by a poly(A)tail, the ribozyme of the hepatitis  $\delta$  virus (HDV) and the termination and polyadenylation sequences of bovine growth hormone (BGH).
31. DNA according to one of claims 22 to 30, wherein the viral sequences are derived from an isolate of the porcine trans-

missible gastroenteritis virus (TGEV), murine hepatitis virus (MHV), infectious bronchitis virus (IBV), bovine coronavirus (BoCV), canine coronavirus (CCoV), feline virus (FCoV), human coronavirus (HCoV), toroviruses or arteriviruses.

32. DNA according to one of claims 22 to 31, wherein the virion is an infectious, non-infectious or replication deficient virus.
33. DNA according to one of claims 22 to 32, wherein the sequence of the a structural or non-structural gene derived from the coronavirus has been modified by substituting, deleting or adding one or several nucleotides of the natural gene sequence.
34. DNA according to one of claims 22 to 33, wherein the sequence of the S, N or M gene has been modified.
35. DNA according to one of claims 22 to 34, wherein the sequence of the S gene derived from a coronavirus has been modified to obtain an attenuated virion.
36. DNA according to one of claims 22 to 35, wherein the sequence of the S gene derived from a coronavirus has been modified to obtain a virion with a tropism differing from the tropism of the coronavirus.
37. Vector comprising a nucleic acid according to one of claims 22 to 36.
38. Vector according to claim 37, wherein the vector is a plasmid or bacterial artificial chromosome (BAC).
39. Host cell comprising a nucleic acid according to one of claims 22 to 38.

40. *E. coli* deposited under CECT 5265 at the Spanish Collection of Type Cultures.
41. Method for producing a recombinant virion or a recombinant viral RNA comprising steps, wherein a DNA according to one of claims 22 to 38 is introduced into a host cell, host cells containing the DNA are cultivated under conditions allowing the expression thereof and the recombinant virion or viral RNA is recovered.
42. Method for producing a recombinant virion or a recombinant viral RNA, wherein a DNA according to one of claims 22 to 38 is mixed with chemicals, biological reagents and/or cell extracts under conditions allowing the transcription of the DNA and the recombinant virion or viral RNA is recovered.
43. Method according to claim 41 or 42, wherein the DNA is a DNA according to one of claims 23 to 38.
44. Virion obtainable by a method according to one of claims 41 to 43.
45. Virion according to claim 44, wherein the virion is an infectious, attenuated, replication defective or inactivated virus.
46. Virion according to claim 44 or 45, wherein the virion comprises a modified S, M or N gene.
47. Virion according to claim 46, wherein modification of the S gene gives raise to an attenuated virus.
48. Virion according to claim 46, wherein modification of the S gene gives raise to a virion with altered tropism.
49. Viral RNA obtainable by a method according to claim 41 to 43.

50. Pharmaceutical preparation comprising a nucleic acid according to one of claims 22 to 38, a host cell according to claim 39 or 40, a virion according to one of claims 41 to 48 or a viral RNA according to claim 49.
51. Vaccine capable of protecting an animal or a human against diseases caused by an infectious agent comprising a nucleic acid according to one of claims 22 to 38, a host cell according to claim 39 or 40, a virion according to one of claims 41 to 48 or a viral RNA according to claim 49.
52. Vaccine according to claim 51, wherein the nucleic acid comprises sequences encoding least one antigen suitable for inducing an immune response against the infectious agent, at least one gene interfering with the replication of the infectious agent or an antibody providing protection against said infectious agent.
53. Vaccine according to claim 51 or 52, wherein said virion vector expresses at least one replication interfering molecule, an antigen capable of inducing a systemic immune response and/or an immune response in mucous membranes against different infectious agents that propagate in respiratory or intestinal mucous membranes or in other tissues.
54. Multivalent vaccine capable of protecting an animal or a human against the infection caused by more than one infectious agent, that comprises more than one nucleic acid according to one of claims 22 to 38, a host cell according to claim 39 or 40, a virion according to one of claims 41 to 48 or a viral RNA according to claim 49, each of which expresses an antigen adequate for inducing an immune response against each of said infectious agents, an interfering molecule or an antibody providing protection against each of said infectious agents.
55. Vaccine according to one of claims 51 to 54 further comprising

sing a pharmaceutically acceptable carrier or diluent.

56. Method of preparing a DNA according to one of claims 22 to 38 comprising steps, wherein an interfering defective genome derived from a coronavirus is cloned under the expression of a promotor into a BAC vector and the deleted sequences within the defective genome are re-inserted.
57. Method of preparing a DNA according to claim 56, wherein toxic sequences within the viral genome are identified before re-insertion into the DNA.
58. Method of preparing a DNA according to claim 56 or 57, wherein the toxic sequences within the viral genome are the last sequences to be re-inserted when completing the genome.
59. An infective clone derived from a coronavirus that comprises a full-length copy of complementary DNA (cDNA) to the genomic RNA (gRNA) of a coronavirus, cloned under a transcription-regulatory sequence.
60. Infective clone according to claim 59, in which said coronavirus is an isolate of the porcine transmissible gastroenteritis virus (TGEV).
61. Infective clone according to claim 59 or 60, in which said promoter is the immediately early (IE) promoter of expression of cytomegalovirus (CMV).
62. Infective clone according to one of claims 59 to 61, wherein said full-length cDNA is flanked at the 3'-end by a poly(A) tail, the ribozyme of the hepatitis delta virus (HDV), and the termination and polyadenylation sequences of bovine growth hormone (BGH).
63. Infective clone according to one of claim 59 to 62, wherein said infective cDNA has been cloned in a bacterial artificial-



al chromosome (BAC).

64. A procedure for obtaining of an infective clone according to any of claims 59 to 63, which comprises constructing the full-length cDNA from the gRNA of a coronavirus and to assembly the transcription-regulatory elements.
65. Procedure according to claim 64, in which the construction of the full-length cDNA of the gRNA of a coronavirus comprises: (i) cloning an interfering defective genome derived from said coronavirus under a promoter of expression in a BAC; (ii) completing the deletions of said interfering defective genome and regenerating the deleted sequences with respect to the infective gRNA; (iii) identifying the toxic sequences for the bacteria in which it is going to be cloned, removing the toxic sequences, and inserting said toxic sequences just before effecting the transfection in eukaryotic cells to obtain the cDNA clone, corresponding to the gRNA of the coronavirus.
66. A recombinant viral vector that comprises an infective clone according to any of claims 59 to 63, or obtainable according to the procedure of either of claims 58 or 59, modified to contain a heterologous nucleic acid inserted into said infective clone under conditions that allow said heterologous nucleic acid to be expressed.
67. Vector according to claim 60, in which said heterologous nucleic acid is selected between a gene and a gene fragment that codes a gene product of interest.
68. A method for producing a product of interest that comprises cultivating a host cell that contains a viral vector according to either of claims 60 or 61 under conditions that allow the heterologous nucleic acid to be expressed and the product of interest to be recovered.

69. A method for producing a modified recombinant coronavirus that contains a heterologous nucleic acid in a sequence of cDNA corresponding to the genome of a coronavirus, which comprises introducing a viral vector according to either of claims 60 or 61 into a host cell, cultivating said host cell containing said viral vector under conditions that allow the viral vector to be expressed and replicated, and the virions obtained from the modified recombinant coronavirus to be recovered.
70. A vaccine capable of protecting an animal against the infection caused by an infectious agent that comprises (i) at least one viral vector according to claim 60 or 61 that expresses at least one antigen suitable for inducing an immune response against said infectious agent, or an antibody that provides protection against said infectious agent, along with, optionally, (ii) a pharmaceutically acceptable excipient.
71. Vaccine according to claim 64, in which said viral vector expresses at least one antigen capable of inducing a systemic immune response and/or an immune response in mucous membranes against different infectious agents that propagate in respiratory or intestinal mucous membranes.
72. A multivalent vaccine capable of protecting an animal against the infection caused by more than one infectious agent, that comprises (i) a viral vector according to claim 60 or 61, that expresses an antigen adequate for inducing an immune response against said infectious agents, or antibodies that provide protection against said infectious agents, along with, optionally, (ii) a pharmaceutically acceptable excipient.
73. A multivalent vaccine capable of protecting an animal against the infection caused by more than one infectious agent, which comprises (i) more than one viral vector accor-

ding to claim 60 or 61, each one of which expresses an antigen adequate for inducing an immune response against each one of said infectious agents, or antibodies that provide protection against each one of said infectious agents, along with, optionally, (ii) a pharmaceutically acceptable excipient.

74. A method for producing a recombinant coronavirus that comprises introducing an infective clone according to any of claims 59 to 63, or obtainable according to the procedure of either of claims 64 or 65 into a host cell, cultivating said host cell that contains the infective clone under conditions that allow the infective clone to be expressed and replicated, and recovering virions obtained from the recombinant coronavirus containing the complete genome of the coronavirus.

## SEQUENCE LISTING

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# PATENT COOPERATION TREATY

From the  
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

## PCT

To:

MENGES, Albrecht v.  
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ALLEMAGNE

UEXKÜLL & STOLBERG

28. MRZ. 2002

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT  
(PCT Rule 71.1)

Date of mailing  
(day/month/year) 27.03.2002

Applicant's or agent's file reference  
P 55171

### IMPORTANT NOTIFICATION

International application No.  
PCT/EP00/12063

International filing date (day/month/year)  
30/11/2000

Priority date (day/month/year)  
03/12/1999

Applicant  
CONSEJO SUPERIOR DE INVESTIGACIONES CIENT... et al

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.
4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

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# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference <b>P 55171</b>	<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. <b>PCT/EP00/12063</b>	International filing date (day/month/year) <b>30/11/2000</b>	Priority date (day/month/year) <b>03/12/1999</b>
International Patent Classification (IPC) or national classification and IPC <b>A61K39/00</b>		
Applicant <b>CONSEJO SUPERIOR DE INVESTIGACIONES CIENT... et al</b>		

1. This International preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.



2. This REPORT consists of a total of 6 sheets, including this cover sheet.

- ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 11 sheets.

3. This report contains Indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☐ Certain observations on the international application

Date of submission of the demand <b>03/07/2001</b>	Date of completion of this report <b>27.03.2002</b>
Name and mailing address of the International preliminary examining authority:  <b>European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax +49 89 2399 - 4465</b>	Authorized officer <b>Tudor, M</b>  Telephone No. +49 89 2399 7700

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/12063

**I. Basis of the report**

1. With regard to the elements of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):  
**Description, pages:**

1-55 as originally filed

**Claims, No.:**

1-74 as received on 25/02/2002 with letter of 20/02/2002

**Drawings, sheets:**

1/13-13/13 as originally filed

**Sequence listing part of the description, pages:**

48-55, as originally filed

2. With regard to the language, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☒ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☐ furnished subsequently to this Authority in computer readable form.
- ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

International application No. PCT/EP00/12063

- ☐ the description,      pages:  
☐ the claims,      Nos.:  
☐ the drawings,      sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Statement**

Novelty (N)	Yes:	Claims	40, 56-74
	No:	Claims	22-24, 26, 28, 32-39, 41-55
Inventive step (IS)	Yes:	Claims	40, 56-74
	No:	Claims	1-21, 25, 27, 29-31
Industrial applicability (IA)	Yes:	Claims	1-74
	No:	Claims	

- 2. Citations and explanations**  
**see separate sheet**

**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT - SEPARATE SHEET**

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International application No. PCT/EP00/12063

**Re Item V**

**Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

**1. Citations**

- 1.1 The documents mentioned in the international preliminary examination report are numbered as in the search report i.e. D1 corresponds to the first document of the search report etc.

**2. Novelty (Article 33(2) PCT) and Inventive Step (Article 33(3) PCT)**

- 2.1 The present application does not satisfy the criterion set forth in Article 33(2) PCT because the subject matter of claims 22-24, 26, 28, 32-39, 41-55 is not new with respect to the prior art as defined in the regulations (Rule 64(1)-(3) PCT).

- 2.2 D4 describes heterologous DNA expression vectors produced from cDNAs corresponding to the genomic sequences of defective transmissible gastroenteritis viruses (TGEV), a coronavirus, and their use in the formulation of vaccines. One embodiment of these vectors is a cDNA derived from a self-replicative RNA containing a heterologous gene encoding the TGEV neutralizing antibody 6A.C3 (see paragraph 0020, example 4, figure 18). Said vector, and virions produced from said vector, also include modifications to the S, N and M gene, are attenuated and have an altered tropism. D4 also discloses methods of producing virions derived from said vector, host cells comprising said vector and said virions, and, pharmaceutical preparations and monovalent or multivalent vaccines containing said vectors or said virions. Therefore, in light of D4 claims 22-24, 26, 28, 32-39, 41-55 cannot be considered novel (Article 33(2) PCT).

3. The present application does not satisfy the criterion set forth in Article 33(3) PCT because the subject matter of claims 1-21, 25, 27, 29-31 does not involve an inventive step (Rule 65(1)(2) PCT).

- 3.1 Document D1, which is considered to be the closest prior art to the subject-matter of claim 1, describes the use of artificial chromosomes, in particular, bacterial



artificial chromosomes (BACs), into which viral DNA sequences have been cloned, for the production of recombinant virus particles both *in vivo* and *in vitro*. Although D1 is directed towards BACs containing DNA encoding the herpes simplex virus (HSV) it is also suggested that members of the RNA viruses, the genomes of which can be made into DNA by standard molecular techniques, can also be employed (page 6, lines 9-13). The difference between D1 and claim 1 is that the applicants have actually cloned the cDNA derived from an RNA virus into a BAC. The apparent technical problem can therefore be considered as to clone the cDNA derived from an RNA virus into a bacterial artificial chromosome. cDNAs derived from RNA viruses, e.g. poliovirus (picornaviridae) vectors, are known in the art (see D5, paragraph 1). Therefore, in order to overcome the apparent technical problem, the skilled person would merely clone a known vector derived from an RNA virus into a BAC, as suggested in D1, and would thus arrive at the subject-matter of claim 1 using standard techniques and without any inventive effort. Therefore, claim 1 does not meet the requirements of (Article 33(3) PCT).

- 3.2 The subject-matter of claims 2-21 represent techniques or choices that would be obvious to the skilled-person faced with the apparent technical problem to be solved and for which no inventive step can be recognised (Article 33(3) PCT).
- 3.3 Claims 25, 27, 29-31 are dependent on the not novel claim 22. The features added by these claims to the subject-matter of claim 22 represent techniques or choices that would be obvious to the skilled-person to overcome the apparent technical problem and for which no inventive step can be recognised (Article 33(3) PCT).
4. Claims 40, and 56-74 satisfy the criterion set forth in Article 33(2) PCT because the subject-matter of said claims is new in respect to the prior art documents as defined in the regulations (Rule 64(1)-(3) PCT). Indeed, none of the cited documents disclose infective clones, bacterial strains containing infective clones or methods for making infective clones, wherein said infective clones comprise a full-length copy of complementary DNA to the genomic RNA of a coronavirus.
- 4.1 Claims 40, 56-74 also satisfy the criterion set forth in Article 33(3) PCT because the subject-matter of said claims shows an inventive step in respect to the prior art

documents as defined in the regulations (Rule 64(1)-(3) PCT)

4.2 D1, which is considered to be the closest prior art to the subject matter of claims 40 and 56-74, discloses DNA encoding a herpes simplex virus cloned into a bacterial artificial chromosome. D1 also suggests that other virus sequences can be employed in the disclosed technique including cDNAs corresponding to the genomic sequence of single plus-stranded RNA viruses e.g. the Coronaviridae (page 6, lines 9-13). However, it appears that before the filing of the present application attempts to clone a full length DNA copy of a coronavirus RNA had proven unsuccessful due the technical problems associated with amplifying a cDNA from an RNA virus with a large genome and to poison sequences within the viral genome which made the cDNA sequence in plasmids unstable. The solution provided by the applicants in the present application of:

- (i) cloning an interfering defective genome derived from said coronavirus under a promoter of expression in a BAC ;
- (ii) completing the deletions of said interfering defective genome and regenerating the deleted sequences with respect to the infective genomic RNA;
- (iii) identifying the toxic sequences for the bacteria in which it is going to be cloned, removing the toxic sequences, and inserting said toxic sequences just before effecting the transfection in eukaryotic cells to obtain the cDNA clone, corresponding to the gRNA of the coronavirus.

is neither disclosed or suggested in the available prior art and is therefore both novel and inventive (Article 33(2) and 33(3) PCT).

## **5. Industrial applicability (Article 33(4))**

5.1 The subject-matter of the present application has industrial applicability.

**Claims**

1. Method of preparing a DNA comprising steps, wherein
  - (a) a DNA comprising a full length copy of the genomic RNA (gRNA) of an RNA virus; or
  - (b) a DNA comprising one or several fragments of a gRNA of an RNA virus, which fragments code for an RNA dependent RNA polymerase and at least one structural or non-structural protein; or
  - (c) a DNA having a homology of at least 60% to the sequences of (a) or (b);is cloned into a bacterial artificial chromosome (BAC).
2. Method according to claim 1, wherein the DNA cloned into the BAC further comprises sequences coding for several or all except one of the structural or non-structural proteins of a virus.
3. Method according to claim 1 or 2, wherein the DNA cloned into the BAC further comprises sequences encoding one or several heterologous gene.
4. Method according to claim 3, wherein the heterologous gene encodes at least one antigen suitable for inducing an immune response against an infectious agent, at least one molecule interfering with the replication of an infectious agent, an antibody providing protection against an infectious agent, an immune modulator, a cytokine, an immoenhancer or an anti-inflammatory compound.
5. Method according to one of claims 1 to 4, wherein the DNA cloned into the BAC has a size of at least 5 Kb.
6. Method according to one of claims 1 to 5, wherein the DNA

- 2 -

cloned into the BAC has a size of at least 15 Kb.

7. Method according to one of claims 1 to 6, wherein the DNA cloned into the BAC has a size of at least 25 Kb.
8. Method according to one of claims 1 to 7, wherein the BAC comprises a sequence controlling the transcription of the DNA cloned into the BAC.
9. Method according to one of claims 1 to 8, wherein one of the genes of the virus has been modified by substituting, deleting or adding nucleotides.
10. Method according to claim 9, wherein the gene controlling tropism of the virus has been modified.
11. Method according to one of claims 1 to 10, wherein the gene controlling tropism of the virus has been substituted with the respective gene of another virus.
12. Method according to one of claims 1 to 11, wherein the DNA cloned into the BAC is capable of being transcribed into RNA which RNA can be assembled to a virion.
13. Method according to of claim 12, wherein the virion is an infectious, attenuated, replication defective or inactivated virus.
14. Method according to one of claims 1 to 13, wherein the virus naturally has a plus strand genome.
15. Method according to one of claims 1 to 14, wherein the virus is a picornavirus, flavivirus, togavirus, coronavirus, torovirus, arterivirus, calcivirus, rhabdovirus, paramixovirus, filovirus, bornavirus, orthomyxovirus, bunyavirus, arenavirus or reovirus.

- 3 -

16. Method of preparing a viral RNA comprising steps, wherein a DNA is prepared according to one of claims 1 to 15, the DNA is expressed in a suitable host cell or the DNA is mixed with chemicals, biological reagents and/or cell extracts under conditions allowing the transcription of the DNA and the viral RNA is isolated.
17. Method of preparing a virion comprising steps, wherein a DNA is prepared according to one of claims 1 to 15, the DNA is expressed in a suitable host cell or the DNA is mixed with chemicals, biological reagents and/or cell extracts under conditions allowing the transcription and translation of the DNA and the virion is isolated.
18. Method according to claim 17, wherein the virion is subsequently inactivated or killed.
19. Method for preparing a pharmaceutical composition comprising steps, wherein a DNA is prepared according to one of claims 1 to 15, a viral RNA is prepared according to claim 16 or a virion is prepared according to claim 17 or 18 and is subsequently mixed with a pharmaceutically acceptable adjuvans or carrier.
20. Method according to claim 19, wherein the pharmaceutical is a vaccine for protecting humans or animals against an infectious disease.
21. Method according to claim 19, wherein the pharmaceutical is used for gene therapy of humans or animals.
22. DNA comprising sequences derived from the genomic RNA (gRNA) of a coronavirus which sequences have a homology of at least 60% to the natural sequence of the virus and code for an RNA dependent RNA polymerase and at least one structural or non-structural protein, wherein a fragment of said DNA is capable of being transcribed into RNA which RNA can be assembled

to a virion.

23. DNA according to claim 22, further comprising a sequence encoding a heterologous gene.
24. DNA according to claim 23, wherein the heterologous gene encodes at least one antigen suitable for inducing an immune response against an infectious agent, at least one molecule interfering with the replication of an infectious agent, an antibody providing protection against an infectious agent, an immune modulator, a cytokine, an immunenhancer or an anti-inflammatory compound.
25. DNA according to claim 22 or 24, wherein said fragment has a size of at least 25 Kb.
26. DNA according to one of claims 22 to 25, which further comprises sequences derived from a coronavirus coding for several or all except one of the structural or non-structural proteins of a virus.
27. DNA according to one of claims 22 to 26, which further comprises sequences derived from a coronavirus coding for all of the structural or non-structural proteins of a coronavirus.
28. DNA according to one of claims 22 to 27, further comprising a sequence controlling the transcription of the viral gRNA.
29. DNA according to one of claims 22 to 28, wherein the sequence controlling transcription of the viral gRNA is the immediately early (IE) promoter of cytomegalovirus (CMV).
30. DNA according to one of claims 22 to 29, wherein the sequence is flanked at the 3'-end by a poly(A)tail, the ribozyme of the hepatitis  $\delta$  virus (HDV) and the termination and polyadenylation sequences of bovine growth hormone (BGH).

31. DNA according to one of claims 22 to 30, wherein the viral sequences are derived from an isolate of the porcine transmissible gastroenteritis virus (TGEV), murine hepatitis virus (MHV), infectious bronchitis virus (IBV), bovine coronavirus (BoCV), canine coronavirus (CCoV), feline virus (FCoV), human coronavirus (HCoV), toroviruses or arteriviruses.
32. DNA according to one of claims 22 to 31, wherein the virion is an infectious, non-infectious or replication deficient virus.
33. DNA according to one of claims 22 to 32, wherein the sequence of the a structural or non-structural gene derived from the coronavirus has been modified by substituting, deleting or adding one or several nucleotides of the natural gene sequence.
34. DNA according to one of claims 22 to 33, wherein the sequence of the S, N or M gene has been modified.
35. DNA according to one of claims 22 to 34, wherein the sequence of the S gene derived from a coronavirus has been modified to obtain an attenuated virion.
36. DNA according to one of claims 22 to 35, wherein the sequence of the S gene derived from a coronavirus has been modified to obtain a virion with a tropism differing from the tropism of the coronavirus.
37. Vector comprising a nucleic acid according to one of claims 22 to 36.
38. Vector according to claim 37, wherein the vector is a plasmid or bacterial artificial chromosome (BAC).
39. Host cell comprising a nucleic acid according to one of

claims 22 to 38.

40. *E. coli* deposited under CECT 5265 at the Spanish Collection of Type Cultures.
41. Method for producing a recombinant virion or a recombinant viral RNA comprising steps, wherein a DNA according to one of claims 22 to 38 is introduced into a host cell, host cells containing the DNA are cultivated under conditions allowing the expression thereof and the recombinant virion or viral RNA is recovered.
42. Method for producing a recombinant virion or a recombinant viral RNA, wherein a DNA according to one of claims 22 to 38 is mixed with chemicals, biological reagents and/or cell extracts under conditions allowing the transcription of the DNA and the recombinant virion or viral RNA is recovered.
43. Method according to claim 41 or 42, wherein the DNA is a DNA according to one of claims 23 to 38.
44. Virion obtainable by a method according to one of claims 41 to 43.
45. Virion according to claim 44, wherein the virion is an infectious, attenuated, replication defective or inactivated virus.
46. Virion according to claim 44 or 45, wherein the virion comprises a modified S, M or N gene.
47. Virion according to claim 46, wherein modification of the S gene gives rise to an attenuated virus.
48. Virion according to claim 46, wherein modification of the S gene gives rise to a virion with altered tropism.



49. Viral RNA obtainable by a method according to claim 41 to 43.
50. Pharmaceutical preparation comprising a nucleic acid according to one of claims 22 to 38, a host cell according to claim 39 or 40, a virion according to one of claims 41 to 48 or a viral RNA according to claim 49.
51. Vaccine capable of protecting an animal or a human against diseases caused by an infectious agent comprising a nucleic acid according to one of claims 22 to 38, a host cell according to claim 39 or 40, a virion according to one of claims 41 to 48 or a viral RNA according to claim 49.
52. Vaccine according to claim 51, wherein the nucleic acid comprises sequences encoding least one antigen suitable for inducing an immune response against the infectious agent, at least one gene interfering with the replication of the infectious agent or an antibody providing protection against said infectious agent.
53. Vaccine according to claim 51 or 52, wherein said virion vector expresses at least one replication interfering molecule, an antigen capable of inducing a systemic immune response and/or an immune response in mucous membranes against different infectious agents that propagate in respiratory or intestinal mucous membranes or in other tissues.
54. Multivalent vaccine capable of protecting an animal or a human against the infection caused by more than one infectious agent, that comprises more than one nucleic acid according to one of claims 22 to 38, a host cell according to claim 39 or 40, a virion according to one of claims 41 to 48 or a viral RNA according to claim 49, each of which expresses an antigen adequate for inducing an immune response against each of said infectious agents, an interfering molecule or an antibody providing protection against each of

said infectious agents.

55. Vaccine according to one of claims 51 to 54 further comprising a pharmaceutically acceptable carrier or diluent.
56. Method of preparing a DNA according to one of claims 22 to 38 comprising steps, wherein an interfering defective genome derived from a coronavirus is cloned under the expression of a promotor into a BAC vector and the deleted sequences within the defective genome are re-inserted.
57. Method of preparing a DNA according to claim 56, wherein toxic sequences within the viral genome are identified before re-insertion into the DNA.
58. Method of preparing a DNA according to claim 56 or 57, wherein the toxic sequences within the viral genome are the last sequences to be re-inserted when completing the genome.
59. An infective clone derived from a coronavirus that comprises a full-length copy of complementary DNA (cDNA) to the genomic RNA (gRNA) of a coronavirus, cloned under a transcription-regulatory sequence.
60. Infective clone according to claim 59, in which said coronavirus is an isolate of the porcine transmissible gastroenteritis virus (TGEV).
61. Infective clone according to claim 59 or 60, in which said promoter is the immediately early (IE) promoter of expression of cytomegalovirus (CMV).
62. Infective clone according to one of claims 59 to 61, wherein said full-length cDNA is flanked at the 3'-end by a poly(A) tail, the ribozyme of the hepatitis delta virus (HDV), and the termination and polyadenylation sequences of bovine growth hormone (BGH).

63. Infective clone according to one of claim 59 to 62, wherein said infective cDNA is cloned in a bacterial artificial chromosome (BAC).
64. A procedure for obtaining of an infective clone according to any of claims 59 to 63, which comprises constructing the full-length cDNA from the gRNA of a coronavirus and to assemble the transcription-regulatory elements.
65. Procedure according to claim 64, in which the construction of the full-length cDNA of the gRNA of a coronavirus comprises: (i) cloning an interfering defective genome derived from said coronavirus under a promoter of expression in a BAC; (ii) completing the deletions of said interfering defective genome and regenerating the deleted sequences with respect to the infective gRNA; (iii) identifying the toxic sequences for the bacteria in which it is going to be cloned, removing the toxic sequences, and inserting said toxic sequences just before effecting the transfection in eukaryotic cells to obtain the cDNA clone, corresponding to the gRNA of the coronavirus.
66. A recombinant viral vector that comprises an infective clone according to any of claims 59 to 63, or obtainable according to the procedure of either of claims 58 or 59, modified to contain a heterologous nucleic acid inserted into said infective clone under conditions that allow said heterologous nucleic acid to be expressed.
67. Vector according to claim 60, in which said heterologous nucleic acid is selected between a gene and a gene fragment that codes a gene product of interest.
68. A method for producing a product of interest that comprises cultivating a host cell that contains a viral vector according to either of claims 60 or 61 under conditions that allow the heterologous nucleic acid to be expressed and the

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product of interest to be recovered.

69. A method for producing a modified recombinant coronavirus that contains a heterologous nucleic acid in a sequence of cDNA corresponding to the genome of a coronavirus, which comprises introducing a viral vector according to either of claims 60 or 61 into a host cell, cultivating said host cell containing said viral vector under conditions that allow the viral vector to be expressed and replicated, and the virions obtained from the modified recombinant coronavirus to be recovered.
70. A vaccine capable of protecting an animal against the infection caused by an infectious agent that comprises (i) at least one viral vector according to claim 60 or 61 that expresses at least one antigen suitable for inducing an immune response against said infectious agent, or an antibody that provides protection against said infectious agent, along with, optionally, (ii) a pharmaceutically acceptable excipient.
71. Vaccine according to claim 70, in which said viral vector expresses at least one antigen capable of inducing a systemic immune response and/or an immune response in mucous membranes against different infectious agents that propagate in respiratory or intestinal mucous membranes.
72. A multivalent vaccine capable of protecting an animal against the infection caused by more than one infectious agent, that comprises (i) a viral vector according to claim 60 or 61, that expresses an antigen adequate for inducing an immune response against said infectious agents, or antibodies that provide protection against said infectious agents, along with, optionally, (ii) a pharmaceutically acceptable excipient.
73. A multivalent vaccine capable of protecting an animal

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against the infection caused by more than one infectious agent, which comprises (i) more than one viral vector according to claim 60 or 61, each one of which expresses an antigen adequate for inducing an immune response against each one of said infectious agents, or antibodies that provide protection against each one of said infectious agents, along with, optionally, (ii) a pharmaceutically acceptable excipient.

74. A method for producing a recombinant coronavirus that comprises introducing an infective clone according to any of claims 59 to 63, or obtainable according to the procedure of either of claims 64 or 65 into a host cell, cultivating said host cell that contains the infective clone under conditions that allow the infective clone to be expressed and replicated, and recovering virions obtained from the recombinant coronavirus containing the complete genome of the coronavirus.

FIGURE 1

FIGURE 1A

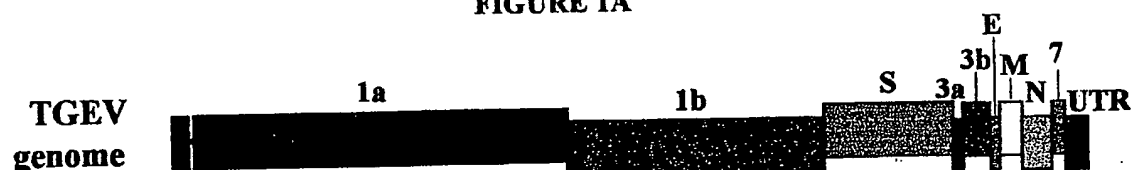


FIGURE 1B

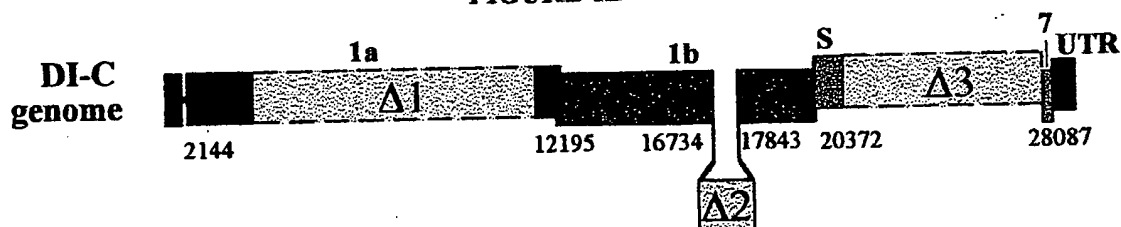


FIGURE 1C

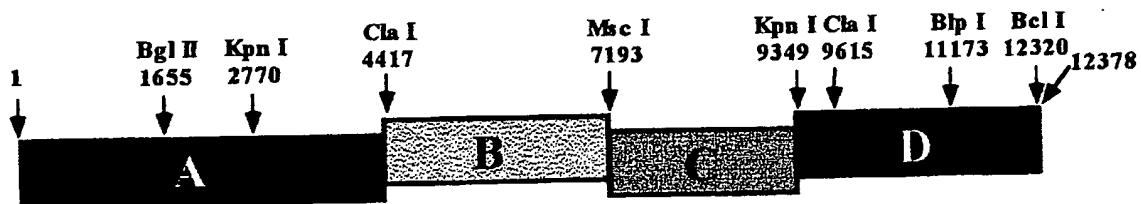


FIGURE 2

# pBelobAC

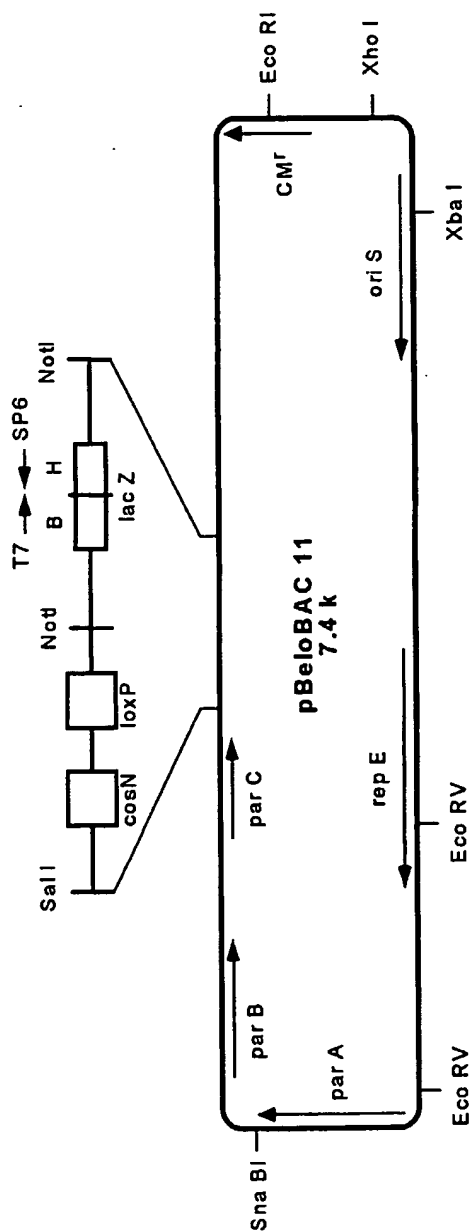






FIGURE 4

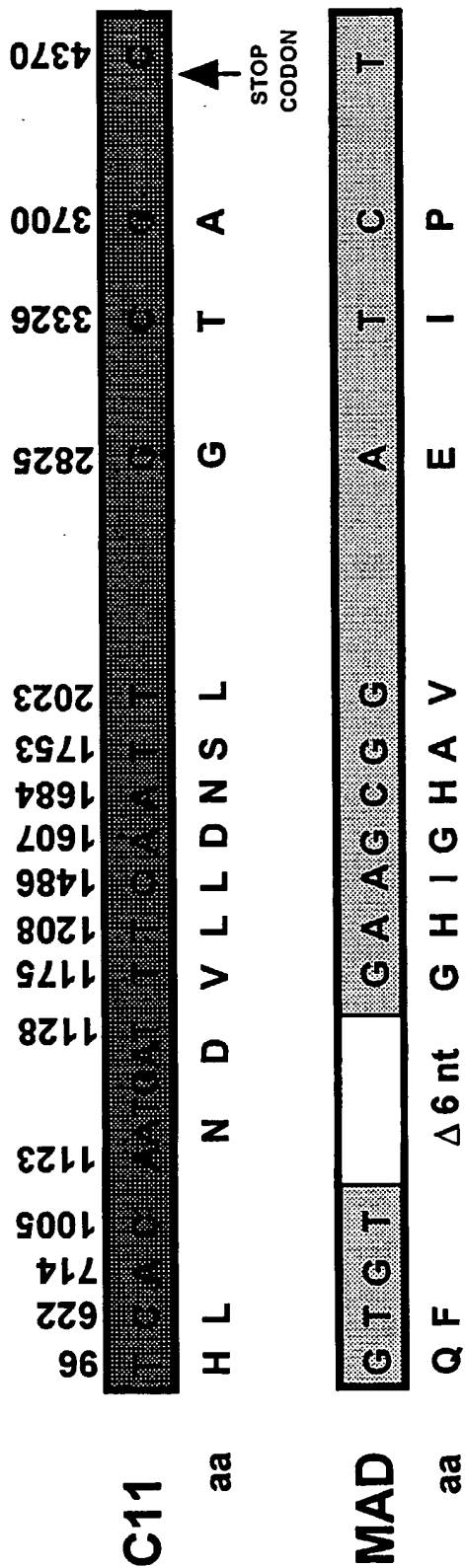
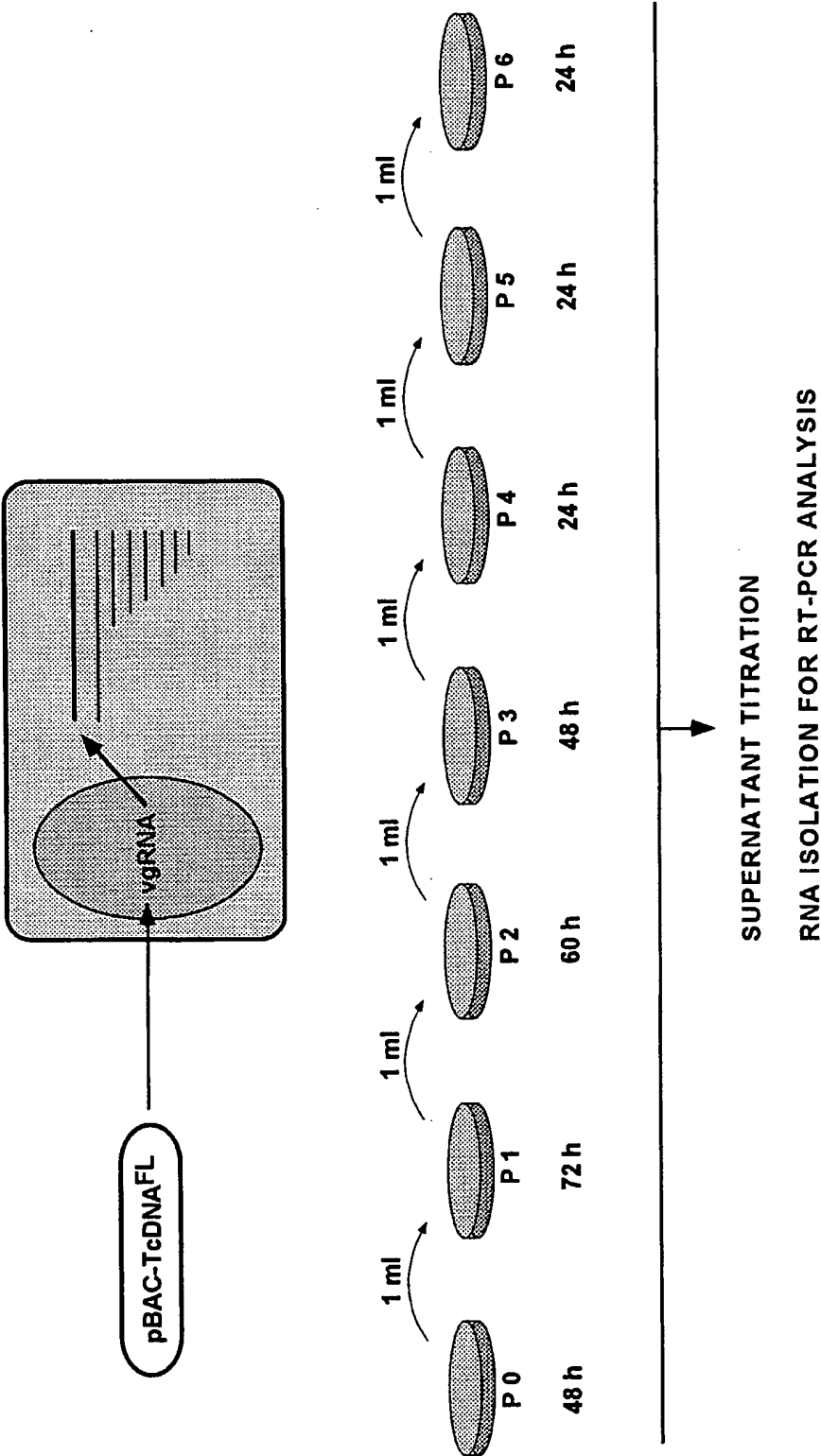
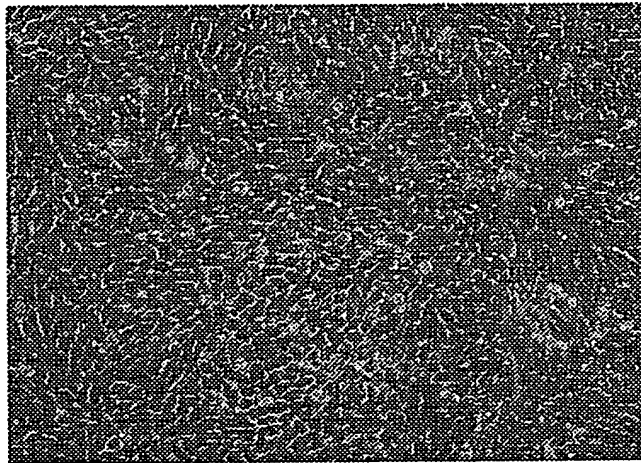


FIGURE 5

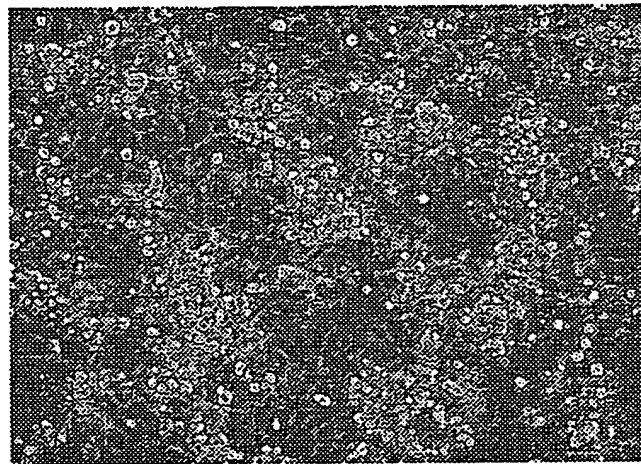


## FIGURE 6

**FIGURE 6A**



**FIGURE 6B**



**FIGURE 6C**

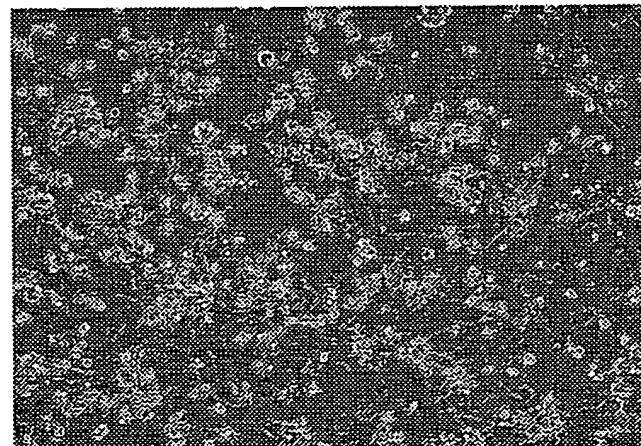
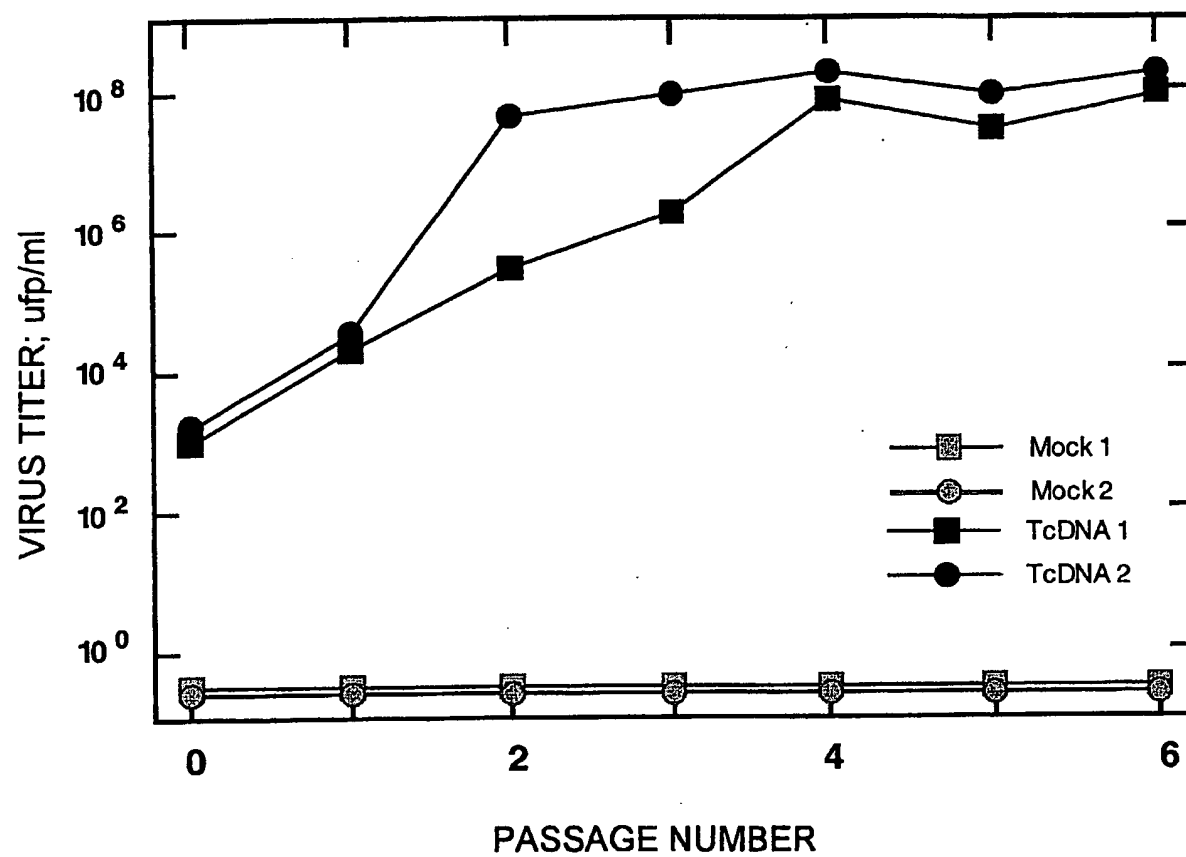


FIGURE 7



## FIGURE 8

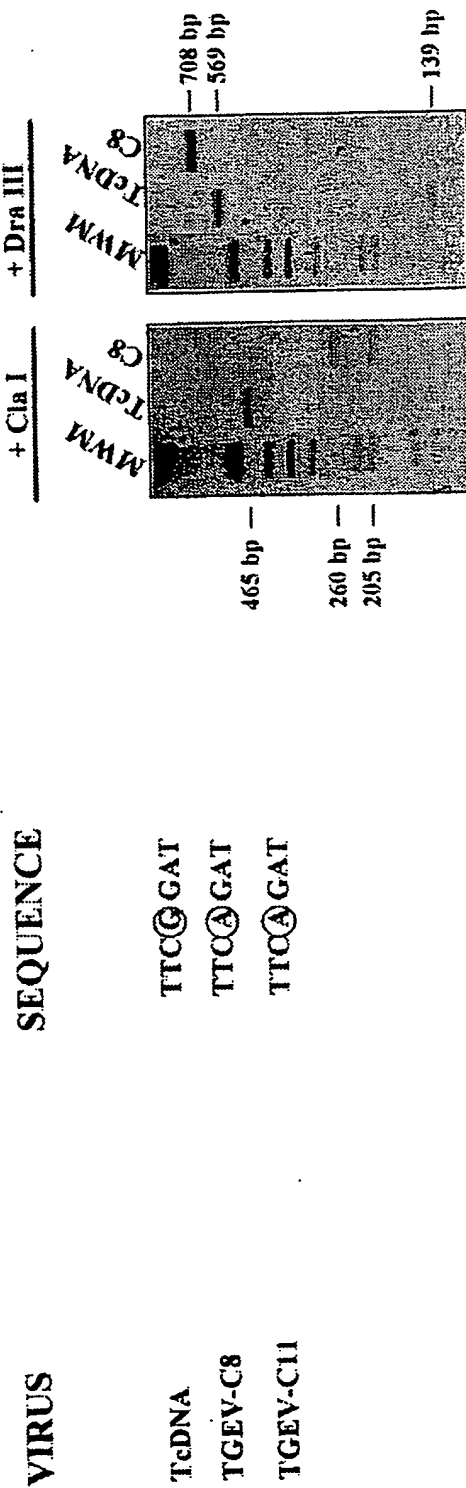
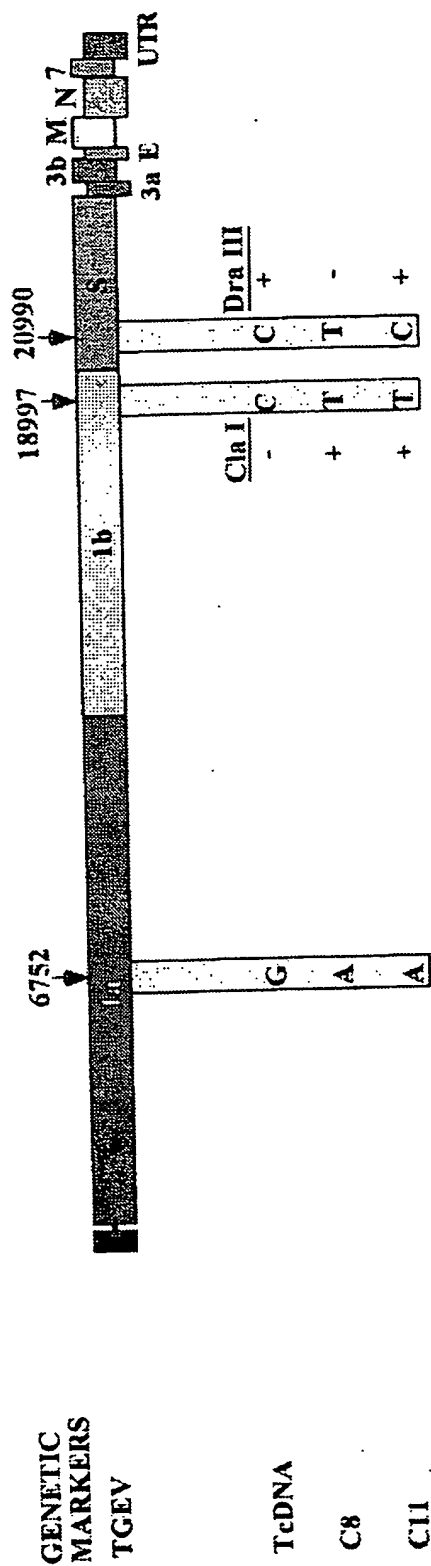
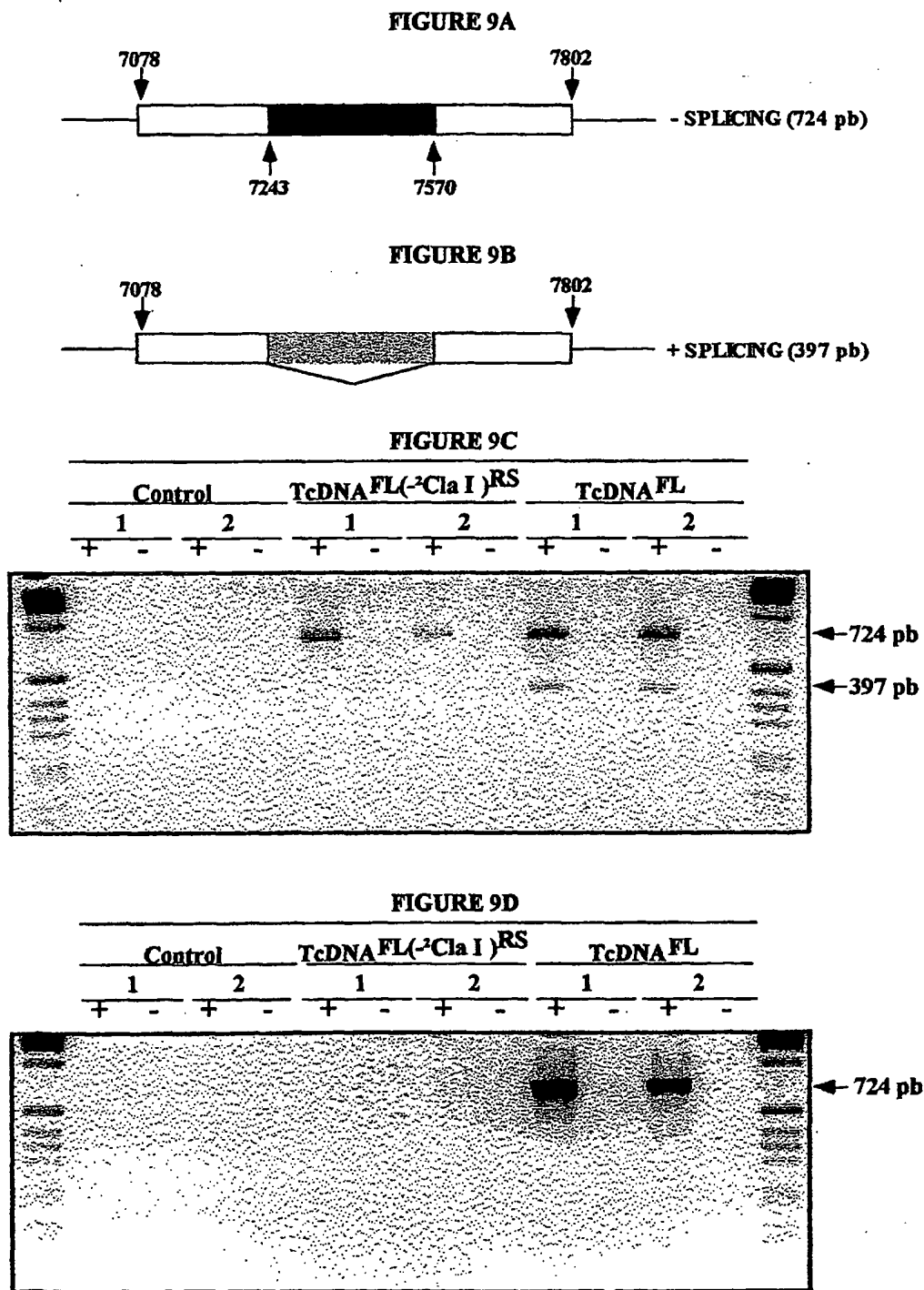
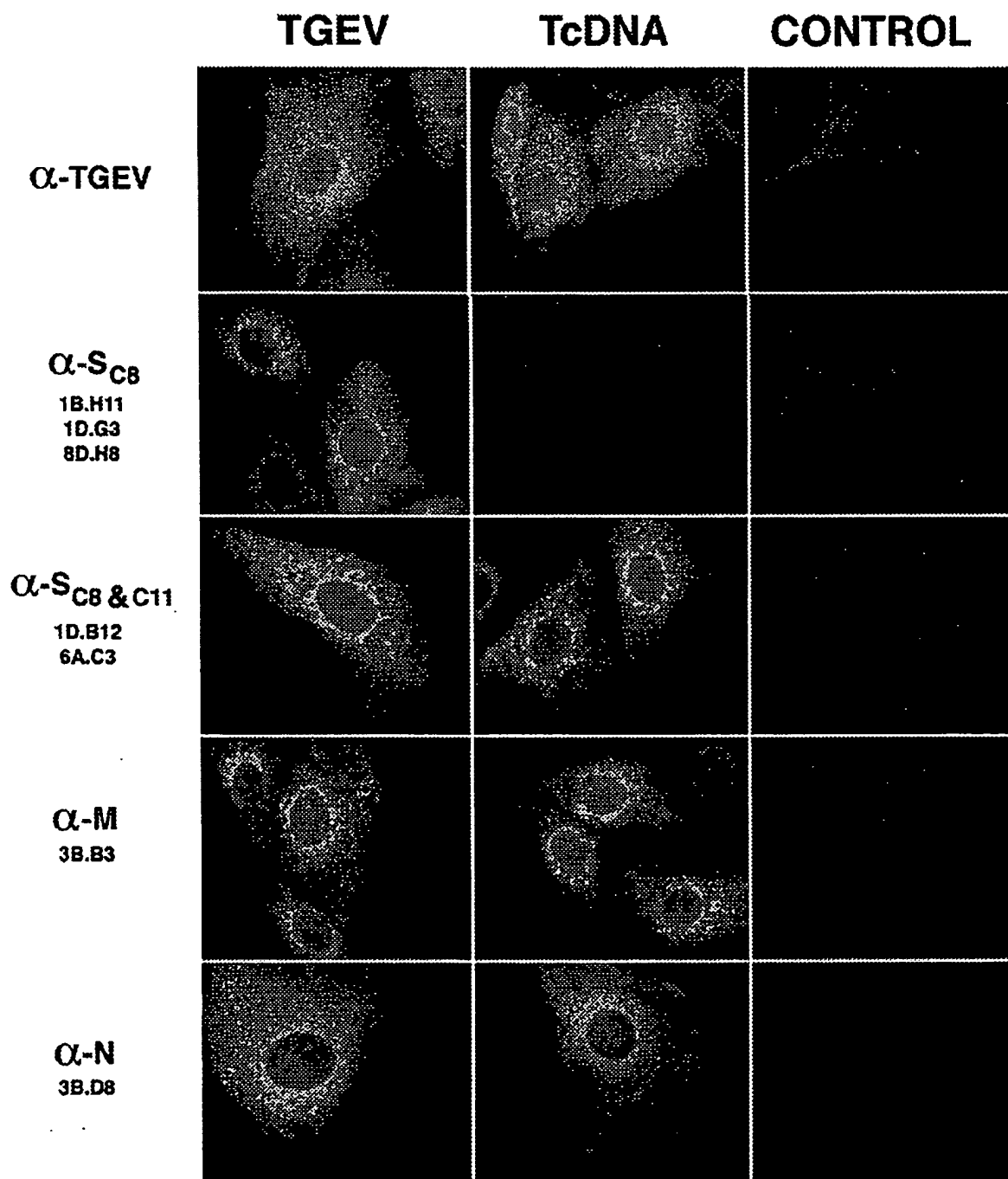


FIGURE 9



**FIGURE 10**

## FIGURE 11

FIGURE 11A

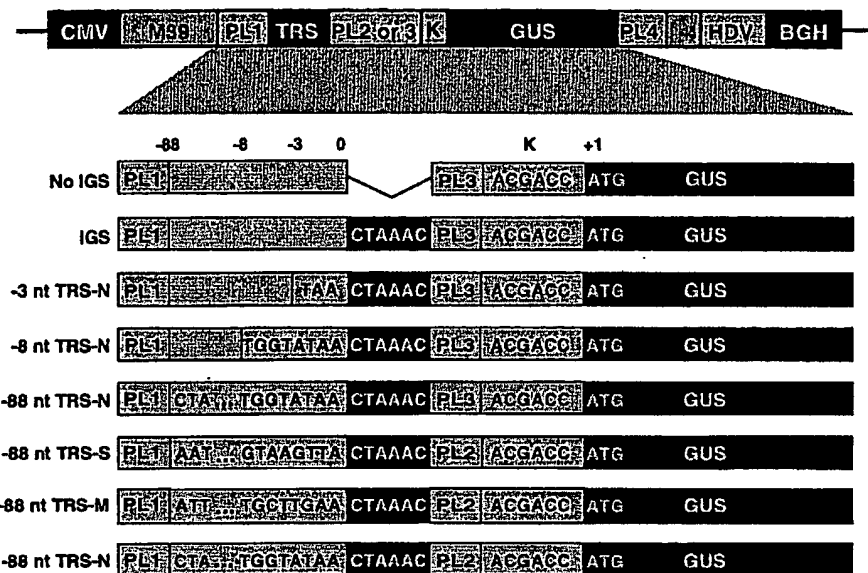


FIGURE 11B

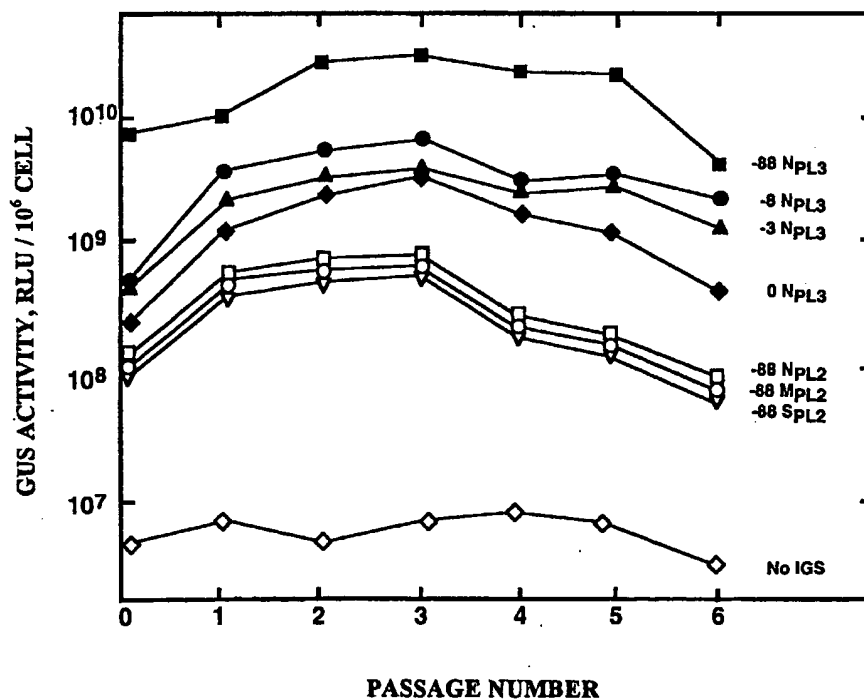




FIGURE 12

FIGURE 12A

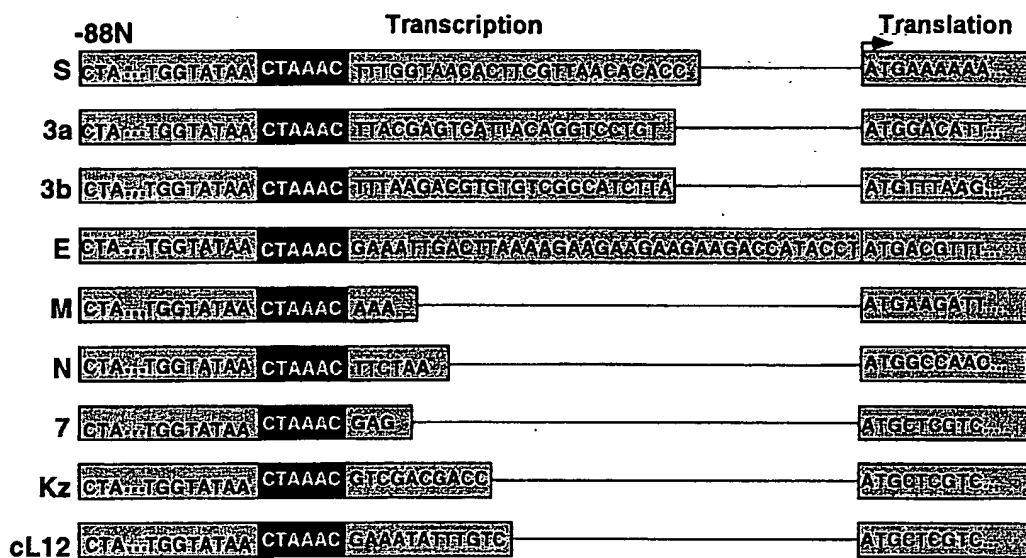


FIGURE 12B

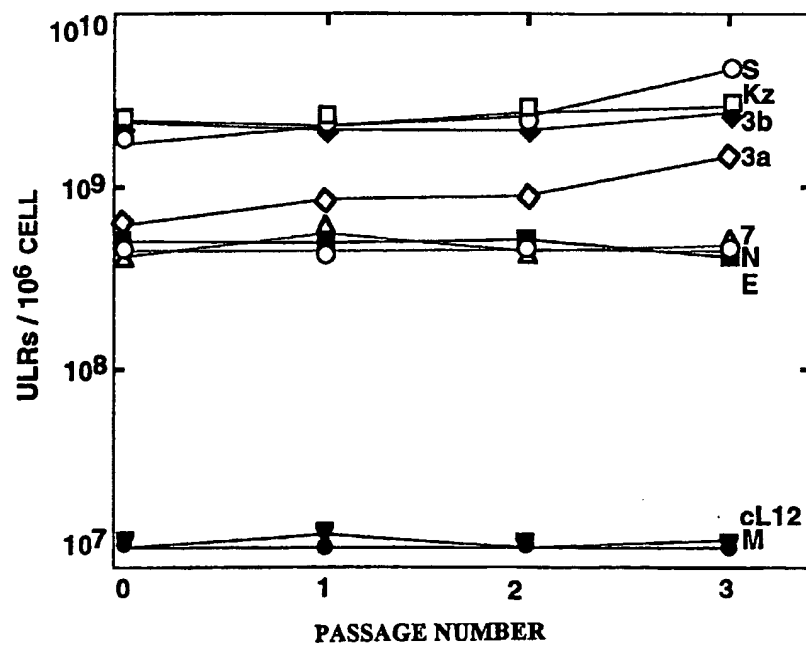


FIGURE 13

FIGURE 13A

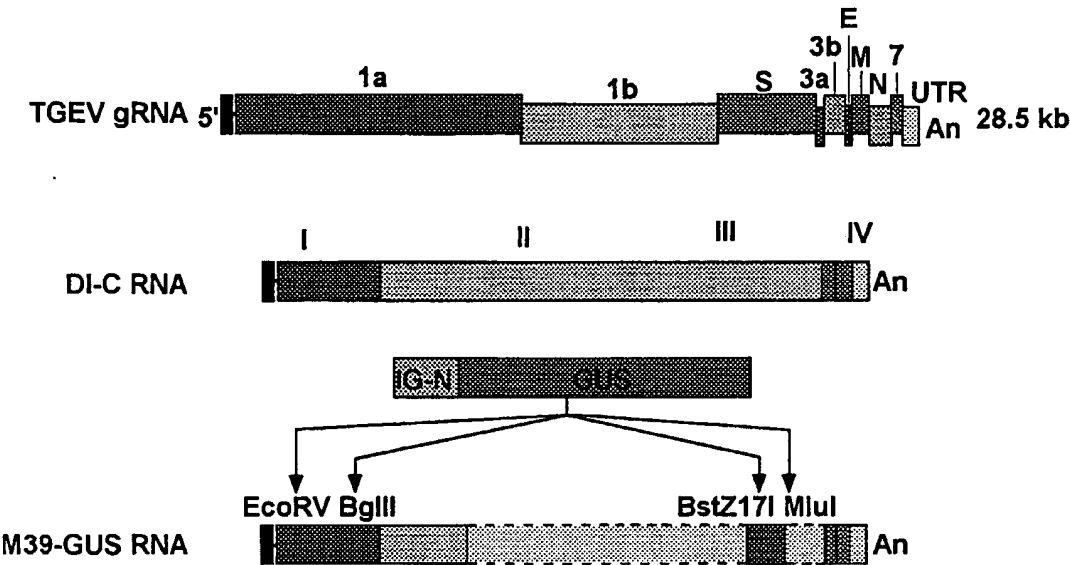
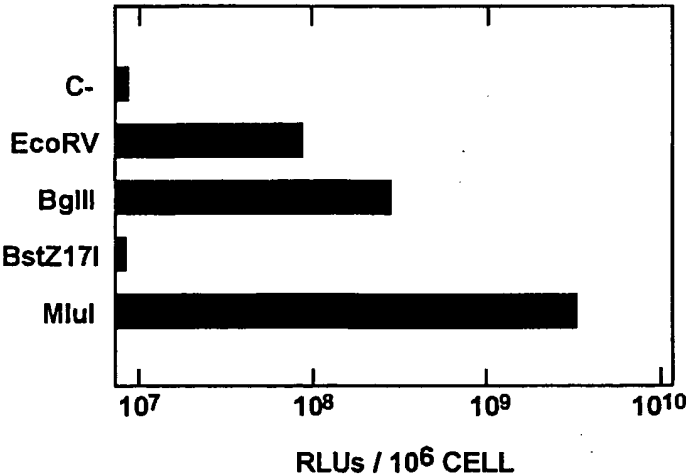


FIGURE 13B



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